J.Q.TAY MBBCh, MRCS

THE ROLES OF VITAMIN D IN CUTANEOUS WOUND HEALING

In vitro and *ex vivo* studies of the effect of 1,25$(OH)_2D_3$ and its precursors on human dermal fibroblasts and epidermal keratinocytes in cutaneous wound healing

Submitted for the Degree of Doctor of Philosophy

Plastic Surgery and Burns Research Unit

Centre for Skin Sciences

University of Bradford

2018

This thesis is dedicated to the 56 who lost their lives in the Bradford City Football Stadium Fire Disaster on 11th May 1985

ABSTRACT

In humans, the epidermis is the main site for the synthesis of Vitamin D_3 (cholecalciferol) from 7-dehydrocholesterol. Cholecalciferol undergoes further hydroxylation in the liver and kidney to produce the active form of the circulating hormone 1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$). In target cells, $1,25(OH)_2D_3$ interacts with the specific intracellular vitamin D receptor (VDR), a member of the nuclear receptor superfamily. However, epidermal keratinocytes, in addition to being target cells, have enzymes required for autocrine production of $1,25(OH)_2D_3$. They can convert cholecalciferol to $1,25(OH)_2D_3$ via 25-hydroxylase (CYP2R1) and 1α-hydroxylase (CYP27B1). Another enzyme, 24-hydroxylase (CYP24A1), regulates local levels by inactivating $1,25(OH)_2D_3$. While recent studies have shown that absence of VDR or $1,25(OH)_2D_3$ impairs formation of granulation tissue during wound healing in mice, little is known about the autocrine and paracrine regulation of biologically active vitamin D_3 by human dermal fibroblasts during cutaneous wound healing.

Primary cultures of human keratinocytes and fibroblasts expressed VDR and all the cytochrome enzymes necessary for autocrine production of vitamin D. The relative expression of VDR mRNA was higher in dermal fibroblasts than donor-matched keratinocytes. In contrast, epidermal keratinocytes had a higher mRNA expression of vitamin D_3 metabolising enzymes. A scratch wound assay confirmed that $1,25(OH)_2D_3$ stimulated keratinocyte migration, but paradoxically inhibited fibroblast migration as early as 4h, yet neither cholecalciferol nor 25-hydroxyvitamin D_3 had any effect. VDR knockdown using small interfering RNA (siRNA) abolished the inhibitory effect of $1,25(OH)_2D_3$ on fibroblast migration, demonstrating the requirement for the VDR in this response.

Immunofluorescent staining revealed that $1,25(OH)_2D_3$ increased nuclear VDR protein expression, without a corresponding increase in VDR mRNA transcription only in mechanically wounded dermal fibroblasts, indicating activation of the receptors. Incubation with either $1,25(OH)_2D_3$, cholecalciferol or $25(OH)D_3$ up-regulated CYP24A1 transcription. This response was most

pronounced with 1,25$(OH)_2D_3$, suggesting a tightly regulated feedback control on 1,25$(OH)_2D_3$ bioavailability within the dermis. In addition, cholecalciferol also increased CYP2R1 and CYP27B1 mRNA expression in scratched dermal fibroblasts, providing evidence for autocrine regulation of 1,25$(OH)_2D_3$ by dermal fibroblasts.

Expression of α-SMA protein was up-regulated in cultured dermal fibroblasts following scratching, which was down-regulated in the presence of 1,25$(OH)_2D_3$. These observations suggest that 1,25$(OH)_2D_3$ may restrict differentiation of wounded dermal fibroblasts into pro-fibrotic myofibroblasts. 1,25$(OH)_2D_3$ also down-regulated MMP-2 secretion and collagen type I to III ratio in scratched dermal fibroblasts. Using a human *ex vivo* wound healing model, it was demonstrated that 1,25$(OH)_2D_3$, but not cholecalciferol, stimulated the rate of wound closure.

In summary, this study has confirmed that human dermal fibroblasts express the transcriptional machinery for autocrine production of 1,25$(OH)_2D_3$, and a higher VDR expression suggests they are more responsive than keratinocytes. Changes in CYP and VDR expression in the presence of cholecalciferol, 25-hydroxyvitamin D_3 or 1,25$(OH)_2D_3$ indicate fine-tuning of the bioavailability of vitamin D in the dermis after wounding. Down-regulation of α-SMA, MMP-2 secretion and the collagen type I to III ratio by 1,25$(OH)_2D_3$ highlight an important role for 1,25$(OH)_2D_3$ in modulating wound healing and the scarring process.

ACKNOWLEDGEMENTS

First and foremost I would like to express my special appreciation and thanks to my PhD supervisor, Dr. M. Julie Thornton. She has been a tremendous mentor for me. I would like to thank her for encouraging my research and for allowing me to grow as a research scientist. I appreciate all her contributions of time and ideas to make my Ph.D. experience productive and stimulating. I would also like to thank Dr. A. M. Graham for being co-supervisor for my research project. The joy and enthusiasm she has for her research was contagious and motivational for me.

Similarly, profound gratitude goes to Irene Castellano Pellicena, a supportive colleague, who is ever so willing to guide me through tough times in the Ph.D. pursuit. She has provided insightful discussions about the research and was instrumental in facilitating my transition as a surgeon into a research scientist. Without her assistance, I would have been lost in the fascinating world of skin sciences.

Thanks are also due to Mr. A.L. Mahajan for giving me the opportunity to undertake my research at the Plastic Surgery and Burns Research Unit. Being able to participate fully in all the fundraising activities in conjunction with the 30th anniversary of Bradford City Stadium Fire has provided me with an invaluable opportunity to meet the amazing people that make the burns unit's work possible and meaningful.

I would also like to thank the laboratory technicians, post-doctorates and academic staffs within the Centre for Skin Sciences for their help and patience during my work in the labs. Finally, I would like to take the opportunity to thank my parents and my family for their unflagging love and unbelievable understanding, without which I could not have accomplished as much as I have.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATION

α-SMA	Alpha-Smooth Muscle Actin
AP	Activator Protein
CBP	Calcium-binding Protein
cDNA	Complementary Deoxyribonucleic Acid
CYP	Cytochrome P450 Enzyme
CYP2R1	Cytochrome enzyme 25-hydroxylase
CYP24A1	Cytochrome enzyme 24-hydroxylase
CYP27B1	Cytochrome enzyme 1α-hydroxylase
DAPI	4',6-Diamidino-2-Phenylindole
DBP	Vitamin D binding Protein
DEJ	Dermal-epidermal Junction
DF	Dermal Fibroblasts
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DBP	Vitamin D Binding Protein
DP	Dermal Papilla
DS	Dermal Sheath
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EK	Epidermal keratinocytes
FAK	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
H&E	Haematoxylin & Eosin
HF	Hair Follicles
IFE	Interfollicular Epidermis
IL-1	Interleukin- 1
IL-6	Interleukin 6

IRS	Inner Root Sheath
KIF	Keratin intermediate filament
LC-MS	Liquid Chromatography-Mass Spectrometry
MAF	Macrophage-activating factor
MAPK	Mitogen-activated protein kinase
MARRS	Membrane-associated, rapid response steroid-binding protein
MMPs	Matrix Metalloproteinases
NCoA	Nuclear Receptor Corepressor
NLS	Nuclear localization sequence
ORS	Outer Root Sheath
^{32}P	Phosphorus-32
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
Pen/Strep	Penicillin/Streptomycin
PFGM	Primary Fibroblasts Growth Media
PFSM	Primary Fibroblasts Starvation Media
PKGM	Primary Keratinocytes Growth Media
PKSM	Primary keratinocytes Starvation Media
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Element
RNA	Ribonucleic Acid
RNAP	RNA polymerase
RXR	Retinoid X receptor
siRNA	Small Interfering RNA
SMRT	Silencing Mediator of Retinoid and Thyroid hormone Receptor
SRC	Steroid Receptor Coactivator

TATA	Thymine Adenine Thymine Adenine
TE	Trypsin/EDTA
TFIIB	Transcription Factor IIB
TFIID	Transcription Factor IID
TGF-β	Transforming Growth Factor-β
TLR2	Toll-like receptor 2
TNF	Tumour Necrosis Factor
UGT1A4	Human uridine 5’-diphophoglucuronyltransferases 1A4
UVB	Ultraviolet light B
VDR	Vitamin D receptor
VDRE	Vitamin D Response Element
1α,25$(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
24,25$(OH)_2D_3$	24,25-dihydroxyvitamin D_3
25$(OH)D_3$	25-hydroxyvitamin D3
25$(OH)D_3$	25-hydroxyvitamin D_3

1 INTRODUCTION

1.1 Structure and function of the skin

The skin is the largest organ of the body, contributing to 6% of total body mass and covering a surface area in excess of 2 m^2 in the adult human (Guyton and Hall 1996; Tobin 2006). It is comprised of three main layers: the epidermis, the dermis and the hypodermis, with each layer exhibiting unique structural and physiological functions (D'Orazio *et al*. 2013; Park 2015). The epidermis and dermis are separated by a dermal-epidermal junction (DEJ). A variety of skin appendages are present in the skin, each serving their own unique functions (Figure 1-1). The skin plays multiple roles in the immune response, provides a mechanical barrier, thermoregulation and sensory functions (Park 2015). Another important function of the skin is in vitamin D_3 synthesis and homeostasis (Mostafa and Hegazy 2015). Its precursor, 7-dehydrocholesterol, which is found mainly in the plasma membrane of epidermal keratinocytes, is converted to vitamin D_3 following UV radiation, before being released into systemic circulation (Mostafa and Hegazy 2015).

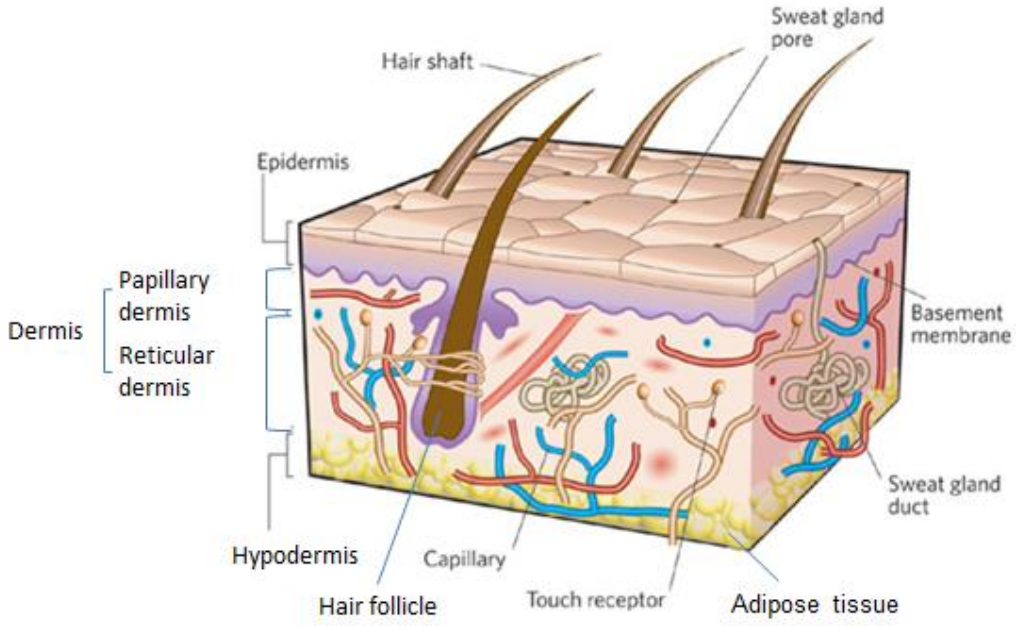


Figure 1-1: **Three main layers of the skin – the epidermis, dermis and hypodermis.** Sweat glands and hair follicles are examples of skin appendages that serve their unique functions in the skin. Adapted from (MacNeil 2007)

1.1.1 The epidermis

The epidermis is an avascular, stratified squamous epithelium that is continually keratinizing, and is normally between 75 and 150 µm thick in human skin (Tobin 2006). The main cell population in the epidermis is the keratinocyte, constituting more than 90% of all epidermal cells (Lulevich *et al*. 2010). The epidermis is derived from embryonic ectoderm (Trounson 2006). The keratinocytes are arranged in five layers, namely the *stratum basale, stratum spinosum, stratum granulosum, stratum lucidum* and *stratum corneum* (Natarajan *et al*. 2014) (Figure 1-2). In the basal layer, keratinocytes are highly proliferative (Fuchs 2008). As they migrate upwards, keratinocytes commit to three distinct differentiation stages (Goldsmith 1991) (Figure 1-2). Eventually, the cornified layer is shed from the skin surface. In human skin, this dynamic flux takes around 28 days to complete, ensuring a continuous supply of inner cells to replace those that are lost at the skin surface (Goldsmith 1991; Braun-Falco 1996; Fuchs 2008). Each stage of differentiation is associated with key

molecular markers such as keratins, loricrin, profilaggrin and involucrin (Braun-Falco 1996) (Figure 1-2).

The keratin intermediate filament (KIF) network contributes to the mechano-resistive strength of the keratinocytes (Lulevich *et al*. 2010). Keratins represent a family of more than 20 polypeptides of 40 to 70 kilodaltons (kDa) in molecular weight (Sun *et al*. 1983; Bragulla and Homberger 2009). The different layers of the epidermis contain a varying amount of keratin, ranging from 30% to 80%, depending on the stage of differentiation (Tobin 2006). Basal cells, which are associated with proliferation, normally express lower molecular weight keratins. On the other hand, the more differentiated keratinocytes express higher molecular weight keratins (Sun *et al*. 1983). Keratin 5 (K5) and Keratin 14 (K14) are commonly used as markers of epidermal cell proliferation, while Keratin 1 (K1) and Keratin 10 (K10) are associated with keratinocyte maturation (Sun *et al*. 1983; Tobin 2006). In addition to roles in maintaining cytoarchitecture, keratins also regulate cell growth, migration, proliferation, apoptosis and organelle transport (Kellner and Coulombe 2009). Keratin 6 (K6) has been implicated in the wound repair process using the murine model (Wong and Coulombe 2003; Rotty and Coulombe 2012). K6-null epidermis showed compromised keratinocyte migration following wounding (Wong and Coulombe 2003).

Other cells found within the epidermis include the melanocytes, Langerhans cells, and Merkel cells (Kidd *et al*. 1971; Boulais and Misery 2008). Melanocytes manufacture, package and transfer melanin to adjacent keratinocytes (Braun-Falco 1996). The amount of melanin present determines the skin colour (Braun-Falco 1996). Melanin has a protective role against UV damage in human skin (Brenner and Hearing 2008). Langerhans cells reside in the basal and suprabasal layers of the epidermis (Chomiczewska *et al*. 2009). They are specialised immune cells that are involved in antimicrobial immunity, skin immunosurveillance and the induction phase of the contact hypersensitivity reaction (Chomiczewska *et al*. 2009). Merkel cells have dense-core granules

which contain heterogeneously distributed neuropeptides (Moll *et al*. 2005). Their main function is to act as slowly adapting mechanoreceptors (Moll *et al*. 2005). This explains their prevalence in touch-sensitive areas (Boulais and Misery 2008).

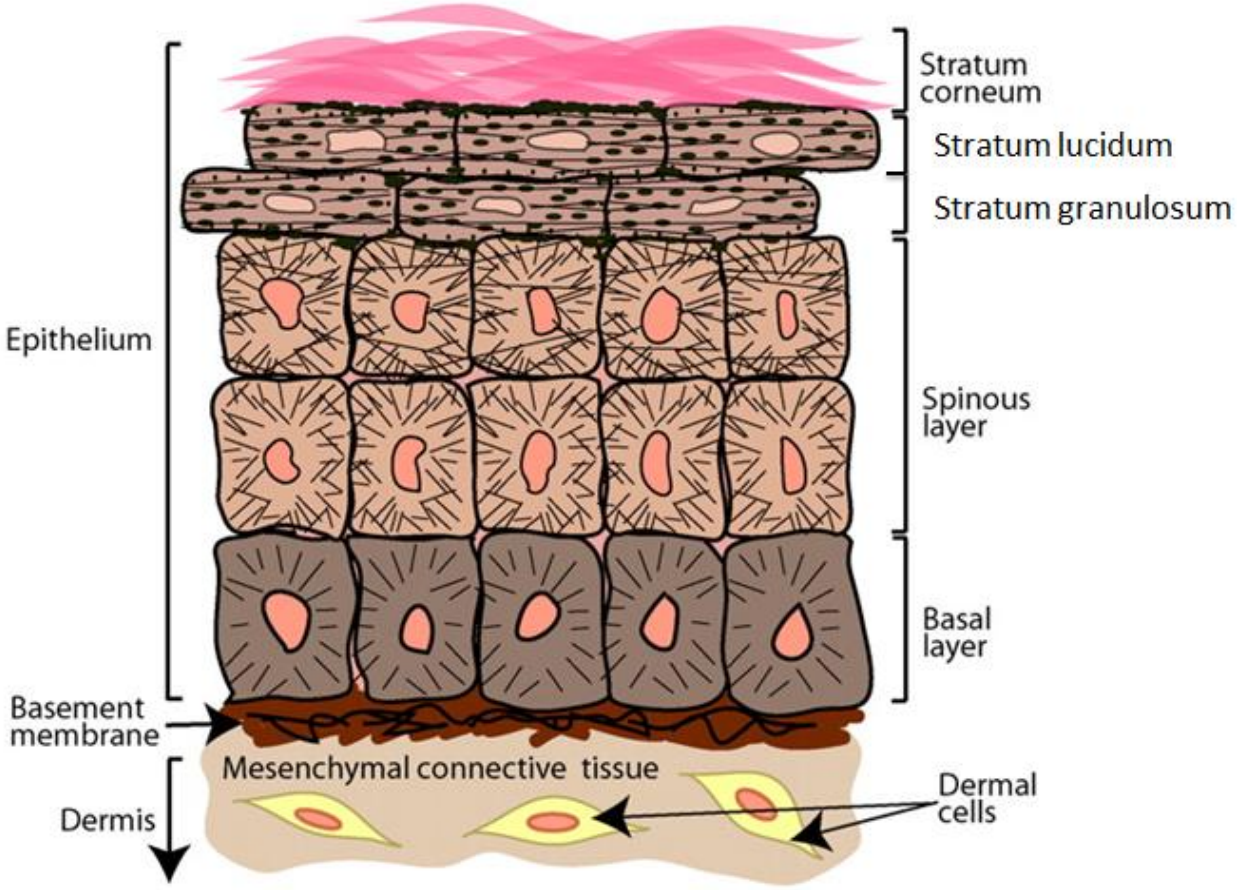


Figure 1-2: Representative image of the epidermis of human skin. The cells at the basal layer are highly proliferative and migrate outwards in their differentiation process. The *stratum corneum*, which is the outermost layer, is mainly constituted of dead keratinocytes. A basal layer of keratinocytes are attached to the basement membrane, which separates them from the mesenchymal connective tissue. Adapted from (Alonso and Fuchs 2003)

1.1.2 The dermis

The main functions of the dermis are to provide mechanical support and nutritional needs to the epidermis (Goldsmith 1991). The dermal layer can be subdivided into an upper 'papillary' and a lower 'reticular' portion (Tobin 2006).

The papillary dermis is more cellular, and relatively thin with less collagen and elastic fibrils than its reticular counterpart. In contrast, the reticular dermis is thicker, with broad bands of dense collagen fibres and long thick interwoven elastin fibres, albeit relatively acellular (Sorrell and Caplan 2004).

The fibroblast is the major cellular constituent of the dermal layer (Thangapazham *et al*. 2014). Fibroblasts are responsible for collagen and glycosaminoglycan synthesis, which contributes to the extracellular matrix (ECM) (Thangapazham *et al*. 2014). They are also involved in removing the ground substance of the ECM, via the action of proteolytic enzymes (Werb and Aggeler 1978). Dermal fibroblasts have the capacity to differentiate into myofibroblasts that generate wound contracture to promote healing (Thangapazham *et al*. 2014). In addition, fibroblasts play a vital role in paracrine and autocrine interactions in skin (Gilchrest *et al*. 1983; Boxman *et al*. 1993; Smola *et al*. 1993; Moulin 1995; Schroder 1995; Smith *et al*. 1997; Werner and Smola 2001; Sorrell and Caplan 2004). Fibroblasts secrete keratinocyte growth factor (KGF)-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 6 (IL-6) and epidermal growth factor (EGF) that regulate keratinocyte proliferation in wound repair (Waelti *et al*. 1992; Boxman *et al*. 1993; Smola *et al*. 1993; Rubin *et al*. 1995; Werner 1998; Breuhahn *et al*. 2000; Mann *et al*. 2001; Werner and Smola 2001).

Lymphocytes, macrophages and mast cells are also present in the dermis. These cells are vital for the inflammatory response and release a variety of growth factors and pro-inflammatory cytokines during the development of granulation tissue (Barrientos et al. 2008). In particular, mast cells that are often found in close proximity to dermal fibroblasts, have been found to modulate fibroblast proliferation in primary human cells in culture (Abe *et al*. 2000). In addition, the dermis houses skin appendages and the vascular, neural and lymphatic systems (Kanitakis 2002; Tobin 2006) (Figure 1-1).

1.1.2.1 Myofibroblast differentiation, matrix metalloproteinases (MMP) activities and collagen content in the dermis

The differentiation of the dermal fibroblast into a myofibroblast is an important process during the wound healing response. Dysregulation of myofibroblast differentiation often leads to abnormal wound healing, which could result in chronic wounds or hypertrophic scarring (Goldberg *et al*. 2007). Alpha-smooth muscle actin (α-SMA) expression within the cytoplasmic stress fibres is the most significant marker for the myofibroblast (Goldberg *et al*. 2007). Morphologically, myofibroblasts are distinguished from “normal” quiescent dermal fibroblasts by the prominence of microfilament bundles in their cytoplasm (Darby *et al*. 2014). When myofibroblasts were first identified, it was suggested that their main role is in contracting the wounds (Gabbiani *et al*. 1971). Since then, the presence of myofibroblasts has been linked to a pathological wound healing response, in which their sustained presence is often a marker of fibrosis and scarring (Darby *et al*. 2014). Myofibroblasts form fibronexus junctions with other cells and also form junctional complexes with the ECM, as a means of transducing mechanical force in the tissue and allowing them to remodel the ECM (Dugina *et al*. 2001; Goffin *et al*. 2006; Darby *et al*. 2014). Apart from their contractile function, myofibroblasts also synthesise and deposit the ECM components in the granulation tissue (see Figure 1-3) (Darby *et al*. 2014). During scar formation, matrix metalloproteinases (MMPs) are involved in progressive remodelling of these granulation tissues (Darby *et al*. 2014). Myofibroblast cell numbers are reduced via apoptosis during the remodelling phase of wound healing (see Figure 1-3) (Darby *et al*. 2014).

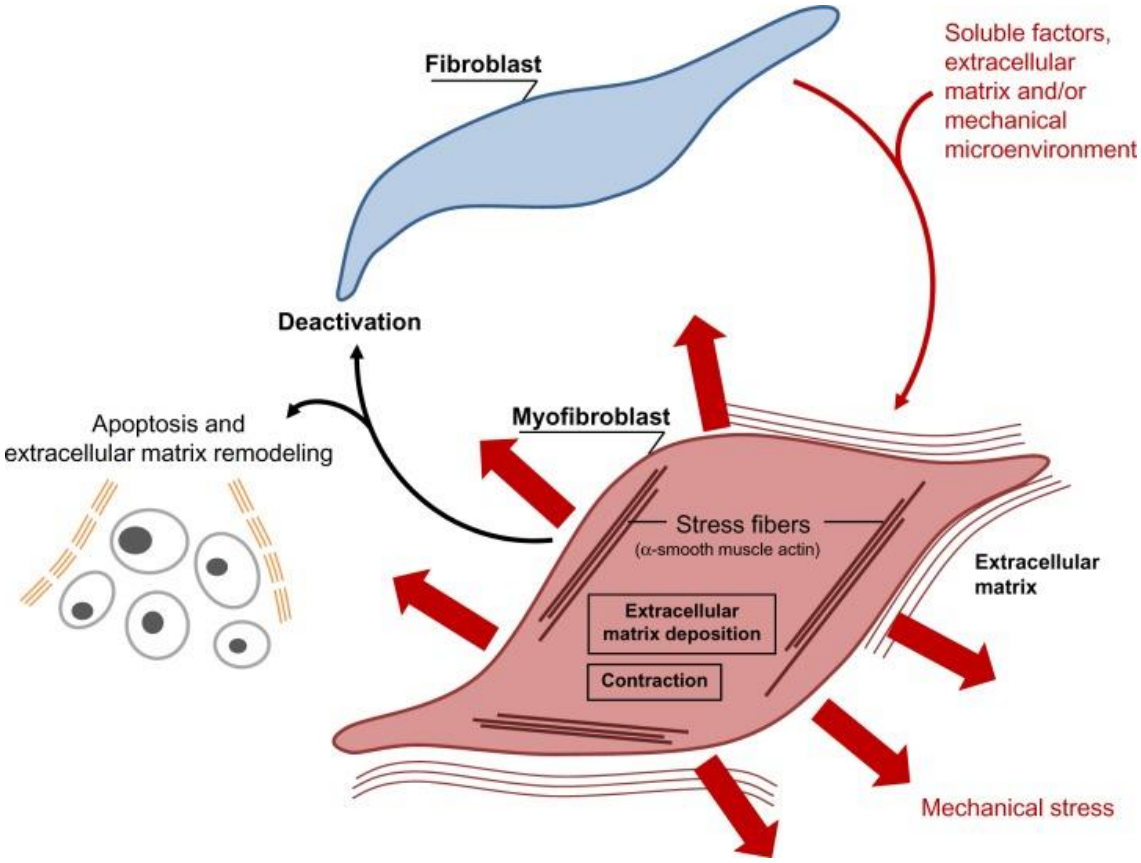


Figure 1-3: The evolution of the myofibroblast phenotype. Fibroblasts are activated and acquire α-SMA expression and differentiate into myofibroblasts. The contractile properties of myofibroblasts are attributed to the α-SMA in microfilament bundles or stress fibres. Myofibroblasts secrete and deposit ECM in granulation tissues. Cell number of myofibroblasts is eventually reduced via apoptosis. Taken from (Darby *et al*. 2014)

Dermal fibroblasts and myofibroblasts play an important role in the deposition of ECM products. They are also responsible for secreting ECM-degrading matrix metalloproteinases (Lindner et al. 2012). Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases, structurally related, which exert catalytic activity against ECM components (Nagase and Woessner 1999; Hieta *et al*. 2003). MMPs can be broadly categorised into soluble MMPs and membrane bound MMPs. Soluble MMPs include gelatinases (e.g. MMP-2, MMP-9), collagenases (e.g. MMP-1, MMP-8), matrilysin (e.g. MMP-7) and stromelysins (e.g. MMP-3, MMP-10) (Lindner et al. 2012). Membrane-type MMPs include MMP-14, MMP-16 and MMP-24 (Lindner et al. 2012). The

structural integrity of the skin, in particular, constituents of the basement membrane, require fine-tuning of the deposition and degradation of ECM components. Hence, MMPs play a central role in tissue remodelling and wound healing.

During wound healing, there is often overlap of MMP substrate specificity and inter-MMP compensation (Caley *et al*. 2015). Some of the MMPs that have been linked to well-characterised phenotypic changes in keratinocytes and fibroblasts during wound healing are summarised in table 1-1. MMPs that have organ specific function and are not related to wound healing are excluded from the table below. An example of this is MMP-20 which is mostly expressed in developing teeth and is important in enamel formation (Vaananen *et al*. 2004; Lu *et al*. 2008). In addition, MMPs in which a functional role in wound healing has not yet been established are also excluded.

Member	***Types (substrate specificity)***	***Role in wound healing***	***Determined in cell culture***		***Reference***
			In vitro	***ex vivo***	
MMP-1	Collagenase	Upregulates human keratinocyte migration	√	√	(Pilcher *et al*. 1997)
MMP-2	Gelatinase	Accelerates human airway epithelial cell migration Induction of breast epithelial cell (HUMEC and MCF10) migration	√		(Giannelli *et al*. 1997; Lechapt-Zalcman *et al*. 2006)
MMP-3	Stromelysin	Promotes re-epithelialization in mice Affecting wound contraction via re-organisation of multicellular actin network in mice	√	√	(Bullard *et al*. 1999)
MMP-7	Matrilysin	Required for re-epithelialization of mice lung mucosal wounds via shedding of syndecan-1 Promotes re-epithelialization in human lung mucosal wound via shedding of E-cadherin ectodomain	√	√	(McGuire *et al*. 2003; Chen *et al*. 2009)
MMP-8	Collagenase	Neutrophil-derived MMP-8 is the predominant collagenase in normal human healing wounds, catalysing breakdown of collagen I, II and III.		√	(Nwomeh *et al*. 1998; Nwomeh *et al*. 1999)

MMP-9	Gelatinase	Promotes human alveolar cell migration and re-epithelialization Promotes human (HaCaT) and mice keratinocyte migration during wound healing. Found at the leading edge of the wound	√	√	(Betsuyaku et al. 2000; Mohan et al. 2002; Seomun et al. 2008; Kyriakides et al. 2009)
MMP-10	Stromelysin	Expressed at epithelial tongue of murine skin wounds. Modestly promotes re-epithelialization with over-expression.		√	(Krampert *et al*. 2004)
MMP-13	Collagenase	Promotes re-epithelialization by upregulating murine epidermal keratinocyte migration, and indirectly by affecting wound contraction		√	(Hattori *et al*. 2009)
MMP-14	Membrane-type	Promotes distal airway re-epithelialization, and keratinocyte migration and proliferation in mice. Promotes murine skin re-epithelialization Important for human keratinocyte (HaCaT, SCC-25) cell migration and proliferation.	√	√	(Nagavarapu *et al*. 2002; Mirastschijski *et al*. 2004; Atkinson *et al*. 2007; Seomun *et al*. 2008)

Table 1-1: Matrix metalloproteinases in wound healing. The MMPs are broadly divided into membrane-bound MMPs and non-membrane bound MMPs (e.g. gelatinase, collagenase, stromelysin, and matrilysin). Adapted from (Chen and Parks 2009) and (Caley *et al*. 2015)

Soluble MMP-2 and MMP-9 both express gelatinolytic activity and are important participants in the wound healing response (Kyriakides et al. 2009). They are involved in the degradation of ECM components such as the basement membrane collagens, thus also facilitate inflammatory cell migration into the injured tissue (Lindner et al. 2012). MMP-2, in particular, is highly expressed by human dermal fibroblasts compared to tissues from other sites of origin (Lindner et al. 2012). MMP-2, also known as getatinase A, exhibits catalytic activity on gelatine, collagen I, IV, V, VII, and X, laminin, aggrecan, fibronectin, vitronectin and tenascin (Chakrabarti and Patel 2005; Caley *et al*. 2015). On the other hand, MMP-9 (i.e. gelatinase B) exerts effects on gelatine, collagen I, III, IV, V and VII, aggrecan, elastin and fibrillin (Caley *et al*. 2015). Their substrate specificities and relatively high expression in human dermal fibroblasts (Lindner et al. 2012), together with their established functional roles in wound healing (see Table 1-1) suggest that they have important roles during the wound healing response. In addition, MMP-2 and MMP-9 levels have been shown to be elevated in chronic wounds, which could lead to chronic tissue high turnover rate and failed wound closure (Wysocki *et al*. 1993). Pro-MMP-2 is readily activated by the membrane-bound MMPs such as MMP-14 and MMP-16 (Murphy and Nagase 2008). On the other hand, MMP-1, MMP-3 and MMP-7 contribute to the activation of pro-MMP-9 into a biologically active form (Murphy and Nagase 2008). The levels of activating enzymes and proenzymes for MMP-2 and MMP-9 also are increased in chronic wounds (Wysocki *et al*. 1993).

Extracellular matrix organisation and remodelling are of paramount importance during the wound healing and scarring process (Xue and Jackson 2015). Collagen, being the most abundant insoluble fibrous protein in the ECM, plays a pivotal role in this process (Lodish H 2000; Xue and Jackson 2015). There are currently more than 16 known human collagen types (Lodish H 2000). Type I, and III collagens contribute to between 80-95 percent of the collagen in the dermis (Meigel *et al*. 1977; Lovell *et al*. 1987; Lodish H 2000; Chung *et al*. 2001; Cheng *et al*. 2011). They exert the most influence on healing tissue integrity, especially during early stages of wound healing, by forming structural scaffolds to enable cell adhesion and migration (Robins *et al*. 2003).

The content and ratio of type I and III collagen in the dermis differs with age and cutaneous injury (Cheng *et al*. 2011). In wound healing, both the level of expression of type I and III collagen, as well as the ratio of type I to III collagen are important determinants of the eventual outcome of the healing response and scarring (Cheng *et al*. 2011). Under basal conditions, type I to III collagen content and its ratio in normal human skin varies across the age spectrum (Cheng *et al*. 2011). With increasing age, relative levels of type III collagen are reduced to a greater degree than type I collagen, which results in an increase in the type I to III collagen ratio (Cheng *et al*. 2011). The ratio is also significantly higher in poor quality wound healing resulting in hypertrophic scarring. In early granulation tissue, type III collagen is normally secreted to a greater extent compared to type I collagen secretion (Haukipuro *et al*. 1991). As the wound contracts and remodelling takes over, immature type III collagen is gradually modified into type I collagen (Slemp and Kirschner 2006). Excessive secretion of type I collagen, with increased type I to III collagen ratio, often result in abnormal hypertrophic scarring (Cheng *et al*. 2011; Shi *et al*. 2013). The biologically active form of vitamin D_3, $1,25(OH)_2D_3$ has been shown to inhibit total collagen synthesis and increase resorption in articular cartilage and osteoblasts (Dickson and Maher 1985; Franceschi *et al*. 1988; Franceschi and Iyer 1992; Li *et al*. 2016). On the other hand, in cultured human neonatal foreskin fibroblasts, $1,25(OH)_2D_3$ has been shown to increase type I and III collagen production after a 4-day exposure to 100nM of $1,25(OH)_2D_3$ (Dobak et al. 1994).

1.1.3 The dermal-epidermal junction and its morphogenesis

The dermal epidermal junction (DEJ) or the basement membrane, establishes the boundary between the epidermis and dermis (Marionnet *et al*. 2006). It is a thin acellular layer composed of type IV collagen, laminin and fibronectin (Kanitakis 2002; Guillaume 2012). The epidermis is avascular, therefore it relies on capillaries in the adjacent papillary dermis to provide it with metabolic support (Yannas 2000). The sub-papillary plexus is an upward projection of capillary loops of the dermis that extends to the dermal-epidermal junction (Yannas 2000) (Figure 1-3). The DEJ is

characterised by rete ridges, i.e. downward folding of the epidermis that interdigitate with the papillary dermis, to maximise the contact area between the two layers (Yannas 2000).

De novo dermal epidermal junction morphogenesis depends on sequential gene expression, protein deposition, and localization of basement membrane zone components (Marionnet *et al*. 2006). During the early phase of the DEJ formation, assembly of laminins through binding to cell membrane receptors, as well as scaffold formation by type IV collagen occur (Fleischmajer *et al*. 1997; Fleischmajer *et al*. 1998). There is tight interplay between fibroblasts and keratinocytes for synthesis and localization of DEJ components (Marionnet *et al*. 2006). Accurate DEJ formation is important due to its fundamental role during wound re-epithelialisation. Its constitutive glycoproteins (e.g. laminins) serve as a substrate for adhesion and migration of keratinocytes (Timpl *et al*. 1979; Breitkreutz *et al*. 2009).

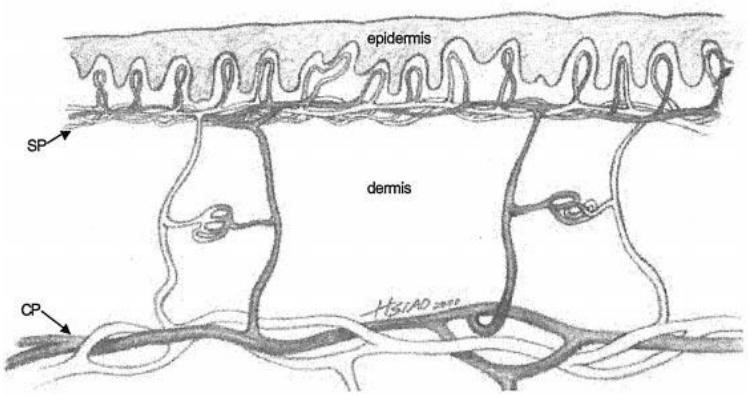


Figure 1-4: Cutaneous and subpapillary plexus supplying the dermis and epidermis. Capillary loops project upwards and extends to the dermal-epidermal junction. Adapted from (Burkitt H. G. 1993). CP denotes cutaneous plexus and SP denotes sub-papillary plexus.

1.1.4 The hypodermis, adipocytes and its relationship with myofibroblasts

The hypodermis underlies the reticular dermis and connects the dermis to the underlying muscles and organs. It contains adipose tissue, blood vessels and nerves. Adipose tissue is important for fat storage and plays important roles in coagulation, glucose and lipid metabolism, angiogenesis, body weight homeostasis, fibrinolysis and vascular tone control (Coelho *et al*. 2013). Until recently, adipocytes found within the adipose tissues were generally regarded as a terminally differentiated cell type and has only been linked to its metabolic functions. However, there is an increasing recognition of the cellular plasticity of adipocytes and their potential roles in the wound healing and scarring response (Bogatkevich 2015; Plikus *et al*. 2017). Marangoni *et al* showed that adipocytes from hypodermal/subcutaneous adipose tissue have the ability to differentiate into myofibroblasts at the early stage of injury (Marangoni *et al*. 2015). The resultant increase in the myofibroblast population could potentially lead to upregulation of collagenous matrix synthesis and worsening fibrosis (Bogatkevich 2015; Marangoni *et al*. 2015). Therapeutic intervention targeting this pathway could help in reducing fibrosis and scarring. More recently, reprogramming of myofibroblasts to form adipocytes was observed when treated with bone morphogenetic protein (BMP) or placed with human hair follicles *in vitro* (Plikus *et al*. 2017). As myofibroblasts are responsible for secretion of profibrotic cytokines, collagen deposition and wound contraction, depletion of myofibroblasts by converting them to adipocytes has been suggested as another possible anti-scarring strategy (Plikus *et al*. 2017).

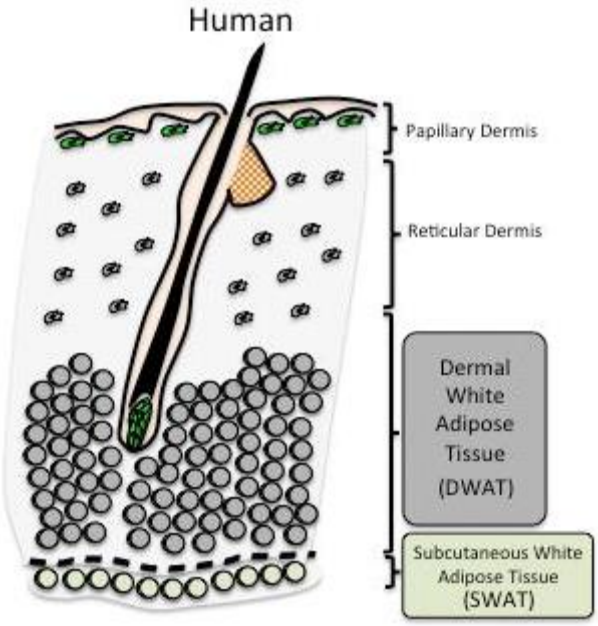


Figure 1-5: Schematic illustration showing white adipose tissue found in the dermis layer and subcutaneous/hypodermis layer. Adapted from (Driskell *et al*. 2014).

1.1.5 Skin appendages

A variety of accessory appendages are found in the skin, each serving their unique functions. These include the secretory and excretory glands, the hair follicles, and nails. Each of these skin appendages serves various physiological functions. Eccrine glands are tubular sweat glands that are not connected to hair follicles and are found only in mammals, whilst apocrine glands generally develop around hair follicles (Kamberov *et al*. 2015). Eccrine glands are important for thermoregulation and are distributed throughout human skin (Kamberov *et al*. 2015). In the context of wound healing, eccrine sweat glands upregulate keratinocyte migration at wound edges that contribute to reepithelialisation (Rittié *et al*. 2013). They also serve as a highly proliferative cell reservoir that could quickly repopulate any wounded area (Rittié *et al*. 2013). Apocrine glands secrete sweat into the pilary canal of the hair follicle and are also important in thermoregulation (Porter 2001).

1.1.5.1 The hair follicles (HF) and hair cycle

The hair follicle is a tubular epithelial structure that can be divided into three segments. The upper segment comprises of the infundibulum, the middle segment

the isthmus, and the lower segment contains the suprabulb and bulb. (Alaiti 2015). The hair follicle is a complex mini-organ that contains the hair shaft, the inner root sheath (IRS), the outer root sheath (ORS), the connective tissue dermal sheath, and the dermal papilla (Figure 1-6) (Schneider *et al*. 2009). Together with the hair, the arrector pili muscle, and sebaceous gland, it makes up the pilosebaceous unit (Sundberg and Nanney 2012).

The hair follicle is made up of a variety of embryologically distinct cell types, which include the keratinocytes, dermal papilla (DP) cells and melanocytes (Randall *et al*. 2003). Ectodermal-mesodermal interactions that are tightly regulated contribute to hair follicle development (Schneider *et al*. 2009). The epithelial components of the hair follicle, the sebaceous glands and the apocrine glands are derived from ectodermal stem cells, whereas the dermal papilla and dermal sheath are derived from the mesoderm (Schneider *et al*. 2009). The follicular dermal papilla is an area of specialised connective tissue that not only regulates hair follicle development and growth, but also acts as a reservoir of multi-potent mesenchymal stem cells (Driskell *et al*. 2011). Dermal sheath (DS) cells share similar characteristics with the dermal papilla cells (Yang and Cotsarelis 2010). The DS serves as a cellular reservoir of DP cells during hair follicle cycling (Yang and Cotsarelis 2010). If the DP is loss, a DS cell has the ability to regenerate a new DP as it contains specialised mesenchymal stem cells (Horne and Jahoda 1992; Reynolds *et al*. 1999; McElwee *et al*. 2003; Yang and Cotsarelis 2010).

The hair follicles are the only permanently regenerating organ in humans (Tobin 2006). Human hair follicles undergo a lifelong repetitive cycling process, alternating between phases of active growth (anagen), regression (catagen), and relative ‘quiescence’ (telogen) (Halloy *et al*. 2002). Human hair follicle cycling is distinctive from other mammals’ in that it cycles asynchronously in different parts of the body (Halloy *et al*. 2000). Initiation of a new hair cycle is induced by subpopulations of epithelial stem cells found in the hair bulge region and the outer root sheath immediately below the bulge of the anagen hair follicle (Chunmeng and Tianmin 2004; Watt and Jensen 2009; Garza *et al*. 2011). These highly proliferative populations of stem cells also contribute to accelerated interfollicular wound healing

by acting as reservoir of epidermal cells for reepithelialisation (Ito *et al*. 2005; Snippert *et al*. 2010; Heidari *et al*. 2016).

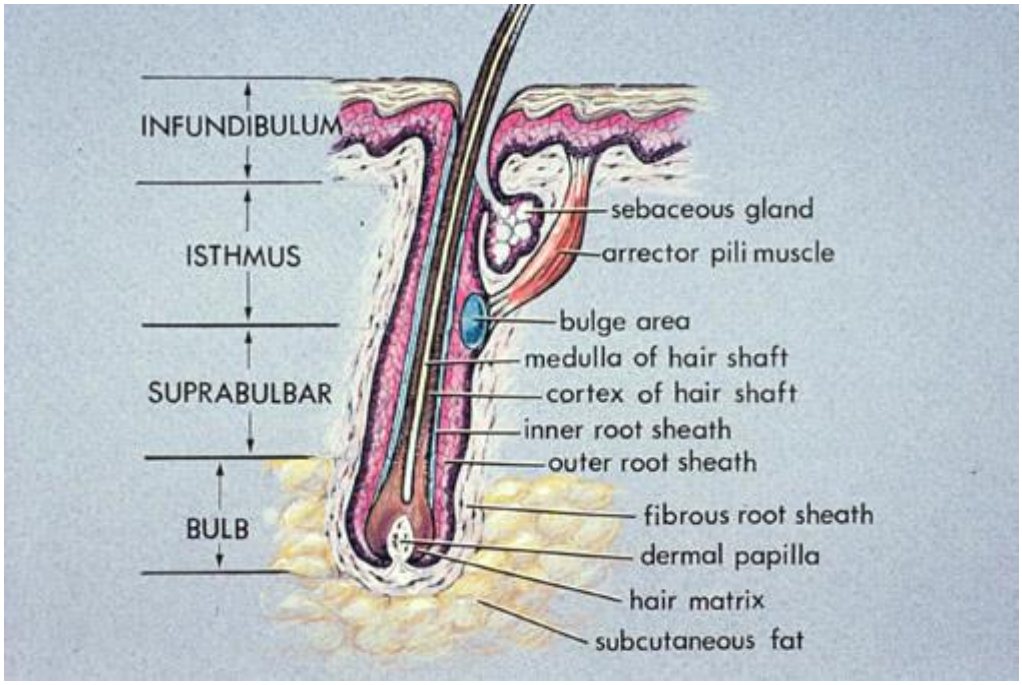


Figure 1-6: Diagrammatic representation of human hair anatomy. The hair follicle is made of an inner root sheath, outer root sheath, dermal sheath, dermal papilla, and bulge area. Hair follicle with the arrector pili muscle and sebaceous gland make up the pilosebaceous unit. Reproduced from (Alaiti 2015)

1.2 Cutaneous wound healing

Adult wound healing serves to restore the continuity of skin barrier following damage to the integrity of the skin. It is a homeostatic response that requires dynamic co-ordination of different cellular and molecular events (Martin 1997). The whole process is complicated, involving blood cells, proteins, proteases, various cytokines, chemokines and growth factors (Sinno and Prakash 2013). Good quality wound healing requires the collaborative efforts of different cell types that are involved in migration, proliferation, differentiation, and apoptosis. Early angiogenesis at the wound site provides the necessary nutrients and oxygen for optimal cellular functions (Li *et al*. 2003). Inflammatory cells are transported to the site of injury to clear away cellular debris and micro-organisms (Li *et al*. 2003). Following haemostasis, wound healing is classically divided into three phases (Gurtner *et al*. 2008) (Figure 1-7).

1.2.1 Haemostasis

Haemostasis is achieved via the actions of platelets and coagulation factors. Endothelial disruption following injury leads to extravasation of blood components into the extravascular space. Reactive vascular spasm serves to restrict the amount of blood loss. Platelet activation and aggregation occurs subsequently to plug the injured vessels and coagulation factors are recruited. Activation of the clotting cascade leads to thrombin production, which catalyses the formation of insoluble fibrin fibres (Golebiewska and Poole 2015). Accumulation of fibrin forms a network of provisional matrix that traps the platelets and binds to growth factors (Shaw and Martin 2009). Activated platelets also secrete fibronectin, cytokines such as TGF-β, and platelet derived growth factor (PDGF), that are all involved in fibroblast proliferation and differentiation, inflammatory cell chemotaxis and angiogenesis (Lynch *et al*. 1987).

1.2.2 The inflammatory phase

Transforming growth factor (TGF)-β1 and β2 secreted by activated platelets, macrophages and keratinocytes are essential chemotactic agents required to initiate the proliferation phase (Sinno and Prakash 2013). Neutrophils are recruited to the wound site via chemotactic migration within 24 hours of wounding. Phagocytosis of bacteria and degradation of necrotic tissues occurs and mediators such as TNF-α, IL-1β and IL-6 are released by neutrophils (Reinke and Sorg 2012), which amplifies the inflammatory response. Neutrophils also release reactive oxygen species (ROS), proteases and matrix metalloproteinases (MMPs) to remove damaged tissues. Monocytes start appearing at the wound site around day 3 after injury and perform further phagocytosis of pathogens and clearance of cell debris (Reinke and Sorg 2012). They remain within the wound site for days to weeks. Other than their phagocytic actions, monocytes also provide a reservoir of growth factors. Lymphocytes arrive in the wound last. They clear away redundant neutrophils and secrete mitogens such as TNF-α (Battegay *et al*. 1995). This leads to fibronectin production and deposition by fibroblasts, initiating the proliferative phase (Stadelmann et al. 1998).

1.2.3 The proliferative phase

Within 24 to 72 hours after injury, the re-epithelialization process starts at the wound edges by epidermal keratinocytes (Reinke and Sorg 2012). Abolition of contact inhibition at desmosomes and hemidesmosomes, coupled with release of different cytokines and growth factors initiates the process (Reinke and Sorg 2012). Fibroblasts and vascular endothelial cells proliferate into the wound to form granulation tissue (Krafts 2010). The newly formed blood vessels are leaky initially, giving rise to the oedematous appearance of the wound. Fibroblasts produce glycosaminoglycans (GAGs) and collagens that are essential to wound repair (Stadelmann et al. 1998). Synthesis and deposition of more collagen occurs to increase the mechanical strength of the wound. Initially, type III collagen is the principal collagen synthesised in the wound (Merkel *et al*. 1988). This is gradually replaced by type I collagen upon entering into the maturation phase of wound healing (Volk et al. 2011). Neovascularisation approaches its maximal point around day 5. Myofibroblast differentiation and consecutive apoptosis reduces the total number of maturing fibroblasts at the wound site (Reinke and Sorg 2012). This stage can last up to 21 days after injury.

Reepithelialisation

Under normal conditions, the basal keratinocytes are anchored to the basal lamina of the basement membrane by hemidesmosomes (Martin 1997). Upon wounding, these hemidesmosome attachments are dissolved by proteinases and new integrins that bind fibronectin and vitronectin are upregulated for migration to occur (Martin 1997). Forward locomotion of basal keratinocytes is directed by intracellular actinomyosin filaments that insert into new adhesion complexes (Mitchison and Cramer 1996). Protrusive structures of the wound-activated keratinocytes contain dense arrays of actin filaments. Filopodia are the simplest protrusive structures that are proposed to take part in keratinocyte migration (Mitchison and Cramer 1996). On the other hand, lamellipodia dominate the leading edges of motile fibroblasts during wound repair (Mitchison and Cramer 1996). Other than the classical actin-based keratinocyte motility, it has been suggested that suprabasal keratinocytes at the leading edge of

the wound may be able to “leapfrog” over basal cells to achieve a similar effect (Garlick and Taichman 1994). This epithelial-mesenchymal phenotype transition of keratinocytes enhances their migratory capacity and invasiveness (Kalluri and Neilson 2003). In hairy skin, stem cells from hair follicles and interfollicular epidermis (IFE) play a vital role in providing progenitor cells for the re-epithelisation process (Ito *et al*. 2005; Snippert *et al*. 2010). Initially, early-arriving IFE stem cells repopulate the wound basal layer (Vagnozzi *et al*. 2015). This is subsequently replaced by late-arriving hair follicle-derived cells (Vagnozzi *et al*. 2015). Hair follicle bulge derived cells are normally constrained at the periphery of the regenerating epithelium (Vagnozzi *et al*. 2015). According to a murine study, hair follicle stem cells contribute to 25-50% of cells in the regenerated epidermis (Levy *et al*. 2007). New stratified epidermis is re-established from the margins of the wound inward (Gipson *et al*. 1988). Epidermal migration ceases once the wound is covered by a monolayer of keratinocytes (Martin 1997).

1.2.4 The remodelling phase

The remodelling phase starts around three weeks after injury, when homeostasis between collagen synthesis and degradation is achieved (Stadelmann et al. 1998). The remodelling process can take up to two years. Degradation of collagen and other extracellular proteins occurs with the aid of matrix metalloproteinases (MMPs). Fibrillar collagens (mostly type I collagen) replace type III collagen to form the major portion of connective tissue in repair sites to optimise the recovery of tensile strength (Reinke and Sorg 2012). Reorganization of collagen fibres, determined by local mechanical factors, leads to a more organized lattice structure (Stadelmann et al. 1998). The number of blood vessels in the wound gradually decreases and cellular activity reduces to normal levels.

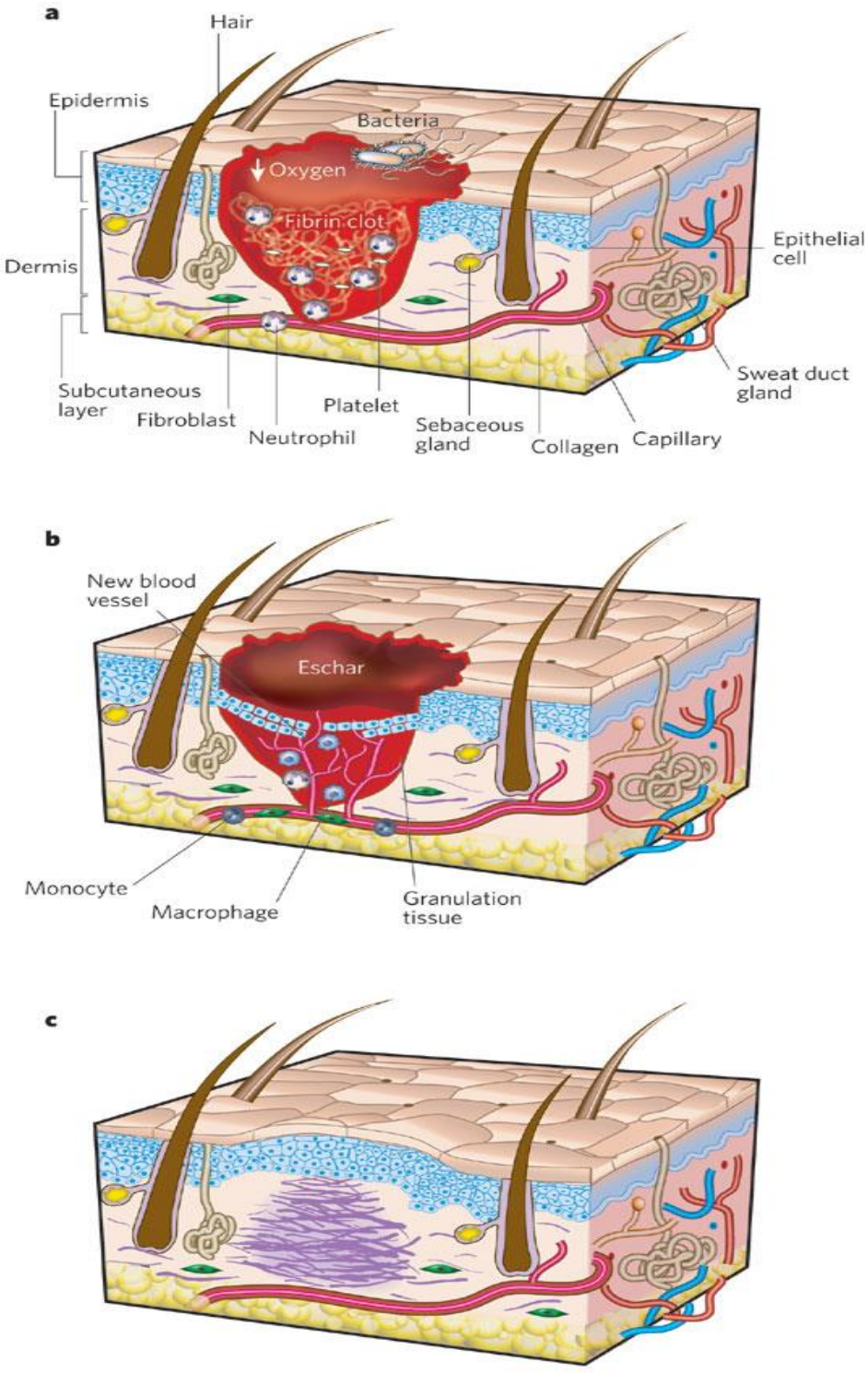


Figure 1-7: The three classical stages of wound repair (A) Inflammation phase (B) Proliferation phase (C) Remodelling phase. There is overlap between the three stages of wound healing. Separate parts of a wound may be at different stages of healing at any one time. Adapted from (Gurtner *et al*. 2008)

1.3 Vitamin D

Vitamin D, also known as calciferol, is a fat-soluble secosteroid prohormone (Ross AC 2011; Jones *et al*. 2014; Mpandzou *et al*. 2016). It was first identified in 1919, when Huldschinsky *et al* noticed that sun exposure was a remedy for rickets (Huldschinsky 1919). Sir Edward Mellanby falsely credited the rickets preventative fat-soluble substance as Vitamin A (Johri 2006). It was later found that it was indeed a new substance, isolated from cod liver oil, and was named by McCollum as Vitamin D (McCollum *et al*. 1922). Its modes of haemostasis and actions are characteristic of a hormone (Norman 2008; DeLuca 2014).

The two main forms of naturally existing vitamin D are: vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) (Nebbioso *et al*. 2015). Cholecalciferol is synthesised in the epidermis following sunlight or ultraviolet (UV) light exposure (Whiting and Calvo 2013; Bendik *et al*. 2014; Herrmann *et al*. 2016). It can also be obtained from food such as salmon and mackerel (Lu *et al*. 2007). On the other hand, ergocalciferol is acquired by consumption of plants or plant materials irradiated with UV (Huang *et al*. 2015). Structural differences exist between ergocalciferol and cholecalciferol. In ergocalciferol, there is a double bond between carbons 22 and 23, and a methyl group on carbon 24 while these are absent in cholecalciferol (Figure 1-8).

The major source of vitamin D for most humans is sunlight exposure (Holick 2008). Cholecalciferol is 2-3 times more effective than ergocalciferol in raising the serum level of 25-hydroxyvitamin D, a major circulating and storage form of vitamin D (Trang *et al*. 1998; Armas *et al*. 2004; Haussler *et al*. 2013; Tripkovic *et al*. 2014; Wilson *et al*. 2014; Oliveri *et al*. 2015). In view of this, discussion in subsequent sections will focus on vitamin D synthesis via the cholecalciferol pathway.

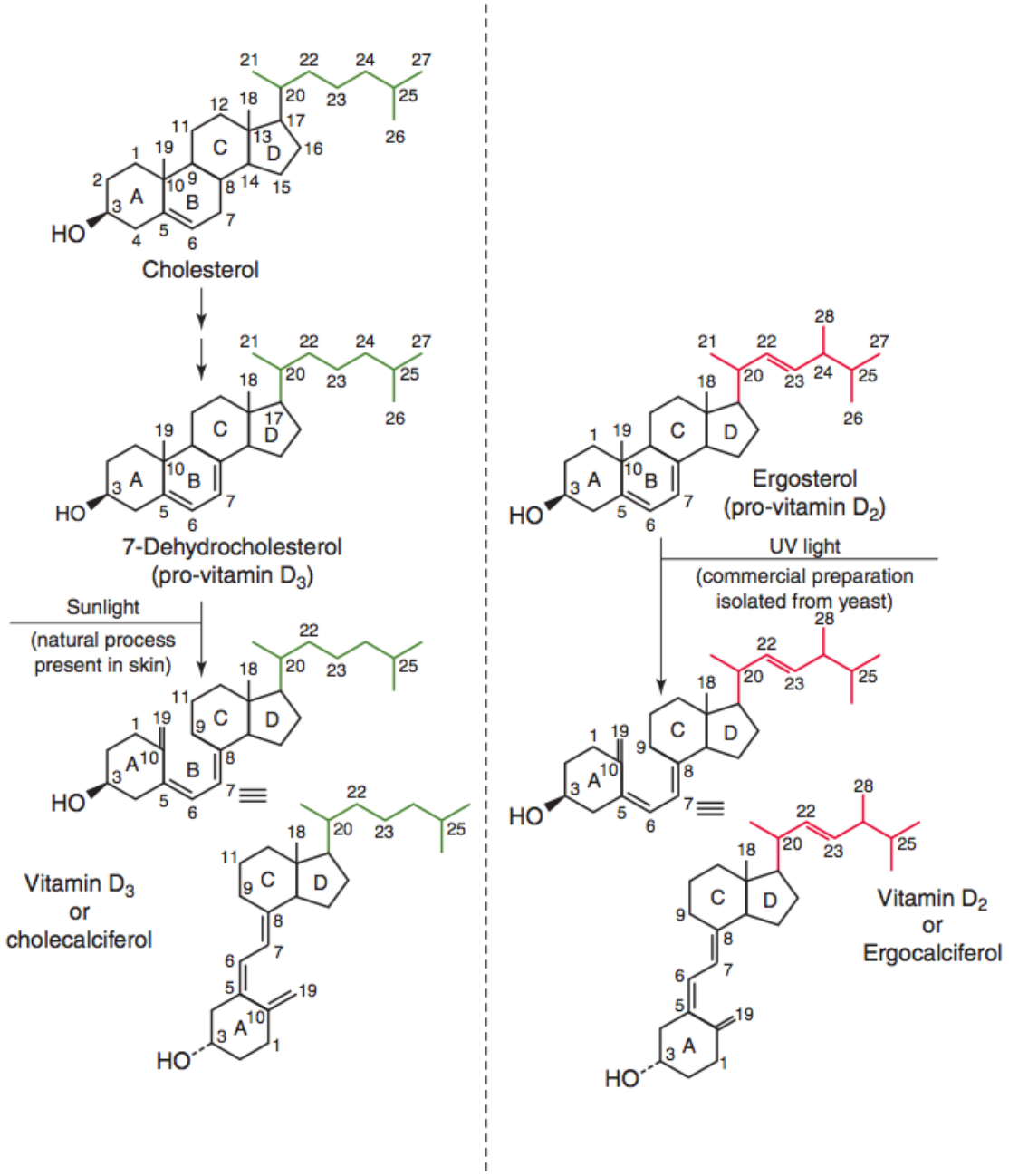


Figure 1-8: Chemical structures of cholecalciferol and ergocalciferol and their precursors. Vitamin D contains three intact rings (A, C and D) with a break between carbon 9 and 10 in the B ring. Cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) differ in the structure of their side chain, with ergocalciferol having a methyl group at carbon 24. Adapted from (Norman 2011)

1.4 Vitamin D metabolism

Vitamin D metabolism involves a variety of precursors, enzymes and metabolites (Bikle 1982; Lindh *et al*. 2012) (Figure 1-9). UVB radiation in sunlight converts 7-dehydrocholesterol, which is found mainly in the epidermis, to cholecalciferol (Anderson *et al*. 2003; Slominski *et al*. 2004; Christakos *et al*. 2010; Whiting and Calvo 2013). Bound to vitamin D-binding protein (DBP), present in the capillary bed of the dermis, cholecalciferol is transported to the liver where a hydroxyl group is added to the 25 carbon position forming 25-hydroxyvitamin D_3 ($25(OH)D_3$) (Anderson *et al*. 2003). $25(OH)D_3$ is the major circulating form of vitamin D_3, bound to DBP and albumin in the bloodstream (Ying *et al*. 2015) (Ohyama and Shinki 2016). A further hydroxylation process takes place in the kidney with the addition of a second hydroxyl group at 1α carbon position, forming the physiologically active form, 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) (Anderson *et al*. 2003).

Cytochrome P450 enzymes, 25-hydroxylase (CYP2R1) and 1α-hydroxylase (CYP27B1) are the main enzymes involved in each of the hydroxylation steps (Christakos *et al*. 2010). The hormonal signal of 1,25-dihydroxyvitamin D_3 is terminated by induction of 24-hydroxylase (CYP24A1) activity (Lindh *et al*. 2012). CYP2R1 is located in the endoplasmic reticulum, whereas CYP27B1 and CYP24A1 are both expressed in the mitochondria (Bikle *et al*. 2014). Oxidative cleavage of the side chain by 24-hydroxylase converts $1,25(OH)_2D_3$ to an inactive metabolite, 1,24,25-trihydroxyvitamin D_3 ($1,24,25(OH)_3D_3$), also known as calcitroic acid (Christakos *et al*. 2010). Alternatively, 23-hydroxylation can occur culminating in a 26,23-lactone by-product (Wei Zheng 2013). CYP24A1 also acts to reduce the pool of $25(OH)D_3$ available for 1α hydroxylation by converting it to $24,25(OH)_2D_3$ (Christakos *et al*. 2010) (Figure 1-9).

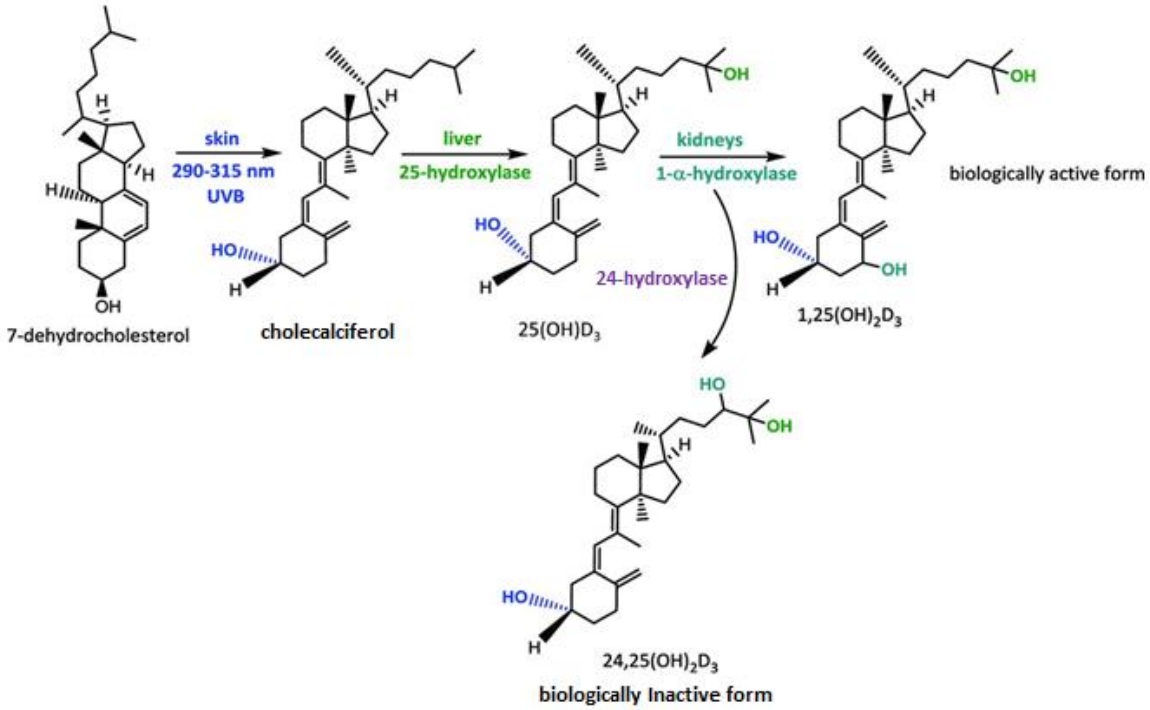


Figure 1-9: Abbreviated metabolic pathway for vitamin D_3 in human skin. High energy UVB radiation breaks the B-ring of the parent steroid between carbon-9 and carbon-10 to form cholecalciferol. CYP2R1 catalyses the addition of a hydroxyl group at carbon 25 (25-hydroxylase) whilst CYP27B1 (1α-hydroxylase) catalyses the addition of a hydroxyl group at position 1α of the secosteroid backbone of 25(OH)D_3. Adapted from (Müller and Volmer 2015)

Several other cytochrome (CYP) enzymes such as CYP27A1 (mammals, murine model), CYP3A4 (human) and CYP2J3 (murine model) have been proposed to have a similar capacity to catalyse the first step of the 25-hydroxylation reaction (Rosen *et al*. 1998; Gupta *et al*. 2004; Prosser and Jones 2004; Aiba *et al*. 2006; Ahn *et al*. 2010; Zhu *et al*. 2013). These provide the possibilities of compensatory pathways for synthesis of 25(OH)D_3. However, studies have shown that the CYP2R1 enzyme is the key vitamin D 25-hydroxylase in humans, with evidence that a genetic defect of CYP2R1 results in an atypical form of vitamin D deficiency clinically (Cheng *et al*. 2004; Zhu *et al*. 2013; Thacher *et al*. 2015). On the other hand, 24-hydroxylase activity has also been detected with CYP3A4 (transfected insect cells model) (Gupta *et al*. 2005). Using high-performance liquid chromatography and gas chromatography–mass spectrometry techniques, Wang *et al* showed that CYP3A4 is capable of carrying out CYP24A1-independent catabolism of 25(OH)D_3 to form 4beta, 25-dihydroxyvitamin D_3, thereby reducing the systemic pool of 25$(OH)_2D_3$ in

humans (Wang et al. 2012; Wang et al. 2013; Wang et al. 2013). Human uridine 5'-diphophoglucuronyltransferases 1A4 (UGT1A4) is an enzyme of the glucuronidation pathway that plays a role in systemic homeostasis of $1,25(OH)_2D_3$. It transforms $1,25(OH)_2D_3$ into water-soluble metabolites for excretion via the enterohepatic pathway (Hashizume *et al*. 2008) (Figure 1-10). The roles of parathyroid hormone and fibroblast growth factor-23 in regulating 1α-hydroxylase expression, thereby affecting bioavailability of $1,25(OH)_2D_3$ is beyond the scope of this project so have been intentionally omitted from discussion (Krajisnik *et al*. 2007; Lavi-Moshayoff *et al*. 2010; Quarles 2012).

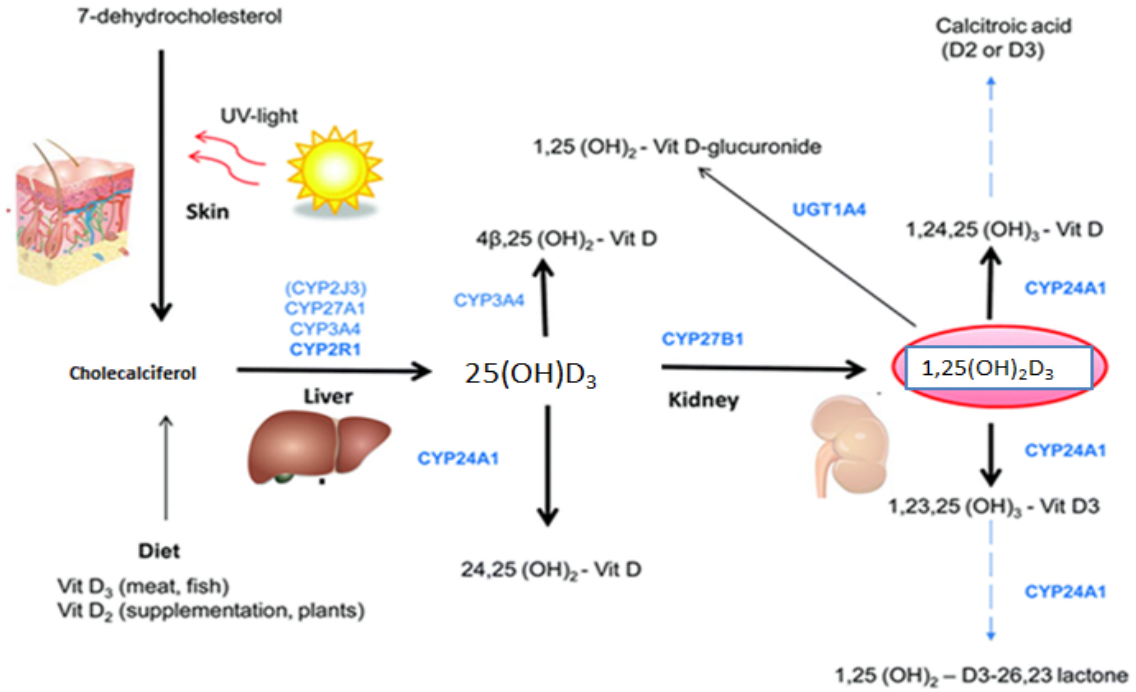


Figure 1-10: Classical vitamin D metabolism pathways. Sunlight exposure and diet are sources of vitamin D in humans. Upon entering the systemic circulation bound to vitamin D transport protein (DBP), cholecalciferol is converted to 25-hydroxyvitamin D_3 by CYP2R1 in the liver. It then undergoes further hydroxylation in the kidney to the active form $1,25(OH)_2D_3$. Excess $1,25(OH)_2D_3$ is inactivated by CYP24A1. Adapted from (Lindh *et al*. 2012)

A novel alternative hydroxylating pathway for the metabolism of vitamin D has been proposed that involves the cytochrome P450 enzyme CYP11A1 (Slominski *et al*. 2013). The products of CYP11A1-mediated metabolism on vitamin D are non-calcemic and non-toxic at relatively high doses and may also serve as partial

agonists of the vitamin D receptors (Slominski *et al*. 2013). It has been shown that human epidermal keratinocytes and dermal fibroblasts can metabolise vitamin D_3 to novel CYP11A1-derived mono-and dihydroxymetabolites, with 20(OH)D3, $20,23(OH)_2D_3$, $20,22(OH)_2D_3$, $17,20(OH)_2D_3$ and $17,20,23(OH)_3D_3$ being the products (Slominski et al. 2014). In human dermal fibroblasts, the major product, which is $20(OH)D_3$, can be further hydroxylated by CYP27B1 to $1,20(OH)_2D_3$ (Slominski *et al*. 2016) (Figure 1-11). Products from this non-classical metabolic transformation of vitamin D_3 could shed light on some of the pleiotropic effects of vitamin D_3, which has previously been attributed only to $1,25(OH)_2D_3$ (Slominski *et al*. 2016).

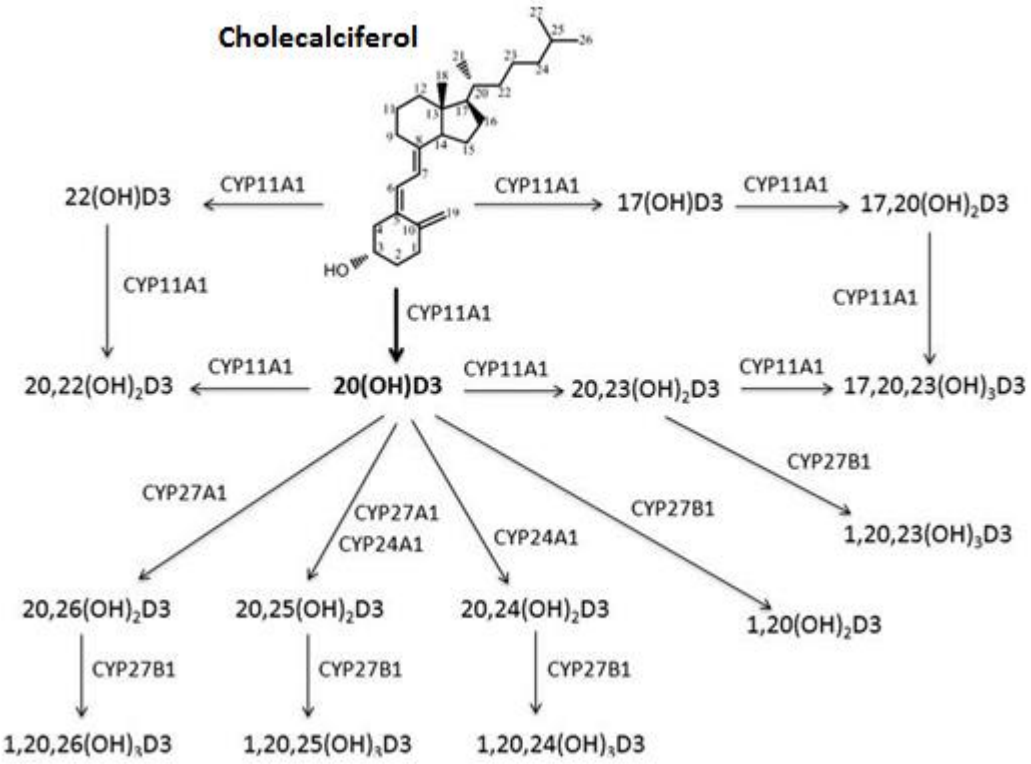


Figure 1-11: Metabolism of cholecalciferol by CYP11A1. CYP27A1, CYP27B1 and CYP24A1 are involved in the further hydroxylation of the metabolites from CYP11A1. Adapted from (Slominski et al. 2014)

1.4.1 Vitamin D binding protein (DBP)

Vitamin D binding protein (DBP) is an albumin-like plasma protein with a molecular mass of approximately 56 kDa (DiMartino *et al*. 2007). The majority of circulating 25-

hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 is tightly bound to vitamin D binding protein (DBP), 10-15% is bound to albumin, and less than 1% of circulating vitamin D exists in an unbound form (Yousefzadeh *et al*. 2014). Besides its role as a high affinity serum transporter, DBP also plays a role in mediating the uptake of DBP-bound $25(OH)D_3$ into some cells capable of converting $25(OH)D_3$ to $1,25(OH)_2D_3$ (Yousefzadeh *et al*. 2014). DBP, in a form bound to a ligand, could have an alternative role as a signal transducer as a result of its cell membrane binding capability (Khan *et al*. 1990; Orchinik *et al*. 1997; Chun *et al*. 2014). Furthermore, DPB has been shown to have the capability to function as a macrophage-activating factor (MAF) and an actin-binder (Sanger *et al*. 1990; Yamamoto and Naraparaju 1996). These effects are independent of its vitamin D metabolite binding functions (Chun *et al*. 2014).

$25(OH)D_3$ binds to DPB with a higher affinity than $1,25(OH)_2D_3$ (Speeckaert *et al*. 2006). Thus, serum $25(OH)D_3$ levels are highly influenced by DBP. This is an important factor to be taken into consideration when defining systemic vitamin D deficiency and providing supplementation (Sollid *et al*. 2016). In the context of target cells that are capable of autocrine production of biologically active vitamin D metabolites, one proposed main function of DBP is to modulate the sensitivity of these cells to $1,25(OH)_2D_3$ (Zella *et al*. 2008; Chun *et al*. 2014). It also limits $1,25(OH)_2D_3$ bioavailability in these cells by regulating the magnitude of peripheral synthesis of $1,25(OH)_2D_3$ via extra-renal 1α-hydroxylase (Chun *et al*. 2014).

1.4.2 Extra-renal production of $1,25(OH)_2D_3$ and local homeostasis of vitamin D by various cell types

Extra-renal production of $1,25(OH)_2D_3$ has been demonstrated in cells such as keratinocytes, macrophages, lymphocytes, osteoblasts and enterocytes (Hewison et al. 2000; Van Etten et al. 2008; Di Rosa et al. 2011). These cells express all the CYP enzymes necessary for $1,25(OH)_2D_3$ production and degradation (van Etten et al. 2008). Tightly regulated vitamin D homeostasis and the presence of vitamin D receptors suggests the important autocrine/paracrine roles of $1,25(OH)_2D_3$ in these cells (van Etten et al. 2008). A few examples of their functions include inhibition of

cell proliferation, promotion of cell differentiation and immune regulation (Dusso *et al*. 2005). These functions are cell type-dependent as a result of different coactivators/corepressor being recruited by the vitamin D receptor (VDR) modulators (Feldman 2011).

1.5 Genomic and non-genomic actions of $1,25(OH)_2D_3$

1.5.1 Vitamin D receptor (VDR), genomic action of $1,25(OH)_2D_3$

Vitamin D Receptor

$1,25(OH)_2D_3$ exerts its genomic actions by binding to the nuclear vitamin D receptor (VDR). Gene expression profiling studies have estimated that VDR signalling is involved in the regulation of up to 5% of human genes (Bouillon *et al*. 2008; Imai *et al*. 2013). The VDR is expressed in cells derived from a variety of organs and tissues (Vuolo *et al*. 2012). These include cardiomyocytes, smooth muscle cells, thyroid C-cells, hair follicle stem cells, T lymphocytes, alveolar epithelial cells, epidermal keratinocytes, dermal fibroblasts, and melanocytes (Vuolo *et al*. 2012). The VDR belongs to a superfamily of nuclear receptors (Evans 1988; Moore *et al*. 2006). It has a molecular mass of approximately 50kDa and consists of 427 amino acids (Jones *et al*. 1998). The human VDR gene is located on chromosome 12q (Deeb et al. 2007). It encodes the full length VDR protein, which includes a DNA binding domain, a nuclear localization domain, a hormone ligand binding domain, a dimerization domain and a transactivation domain (Deeb et al. 2007) (Figure 1-12).

The VDR functions as a ligand-activated transcription factor (Thompson *et al*. 1998). It is translocated into the nucleus by moving along microtubule tracts to the nuclear pores, directed by nuclear localization signals (Deeb et al. 2007). Its ligand binding domain has a high affinity for $1\alpha,25(OH)_2D_3$ ($Kd=10^{-10}M$), but not for cholecalciferol (Thompson et al. 1998; Ohyama and Shinki 2016). Activation by $1\alpha,25(OH)_2D_3$ results in a conformational change to the VDR which strongly recruits the retinoid X receptor (RXR) to form a heterodimer (Thompson et al. 1998; Deeb et al. 2007) (Figure 1-13). The $1,25(OH)_2D_3$-VDR-RXR complex binds to vitamin D response elements (VDREs) at the DNA-binding domain of target genes (Deeb et al. 2007).

The VDR can also bind to the basal transcription factor II B (TFIIB) via protein-protein interactions (Haussler *et al*. 1997) (Figure 1-12). An amphipathic alpha-helix domain, VDR activation function-2 domain (AF-2) transactivation domain is positioned at the extreme C-termini of both the VDR and RXR (Figure 1-12). Interaction of AF2 with stimulatory co-activators such as steroid receptor coactivators is a pre-requisite for downstream transcriptional activity (Haussler et al. 1997; Deeb et al. 2007). The VDR, RXR and TFIIB complex, coupled with the RNA polymerase II transcription machinery leads to transcriptional initiation of VDRE-containing, vitamin D target genes (Haussler *et al*. 1997) (Figure 1-13).

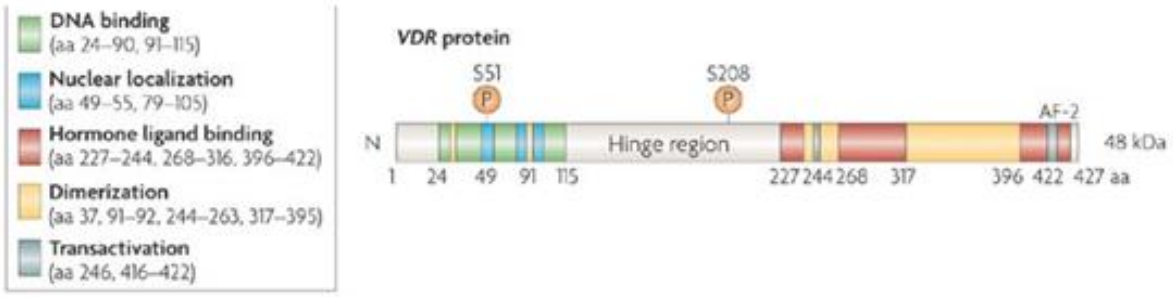


Figure 1-12: VDR protein with its five functional domains that mediate transcriptional activation of target genes. The VDR is stabilised by phosphorylation of serine 51 in the DNA-binding domain by protein kinase C and serine 208 in the hinge region. VDR localization signals direct the receptor into the nucleus. Upon binding of 1,25$(OH)_2D_3$ to the hormone ligand binding domain, the VDR associates with the retinoic acid receptor (RXR) though its dimerization domain. The 1,25$(OH)_2D_3$-VDR-RXR complex then binds to vitamin D response elements (VDREs) through the DNA-binding domain. This results in a conformational change in the VDR that allows the interaction of the VDR activation function 2 (AF2) transactivation domain with stimulatory coactivators. Adapted from (Deeb et al. 2007). aa denotes amino acids.

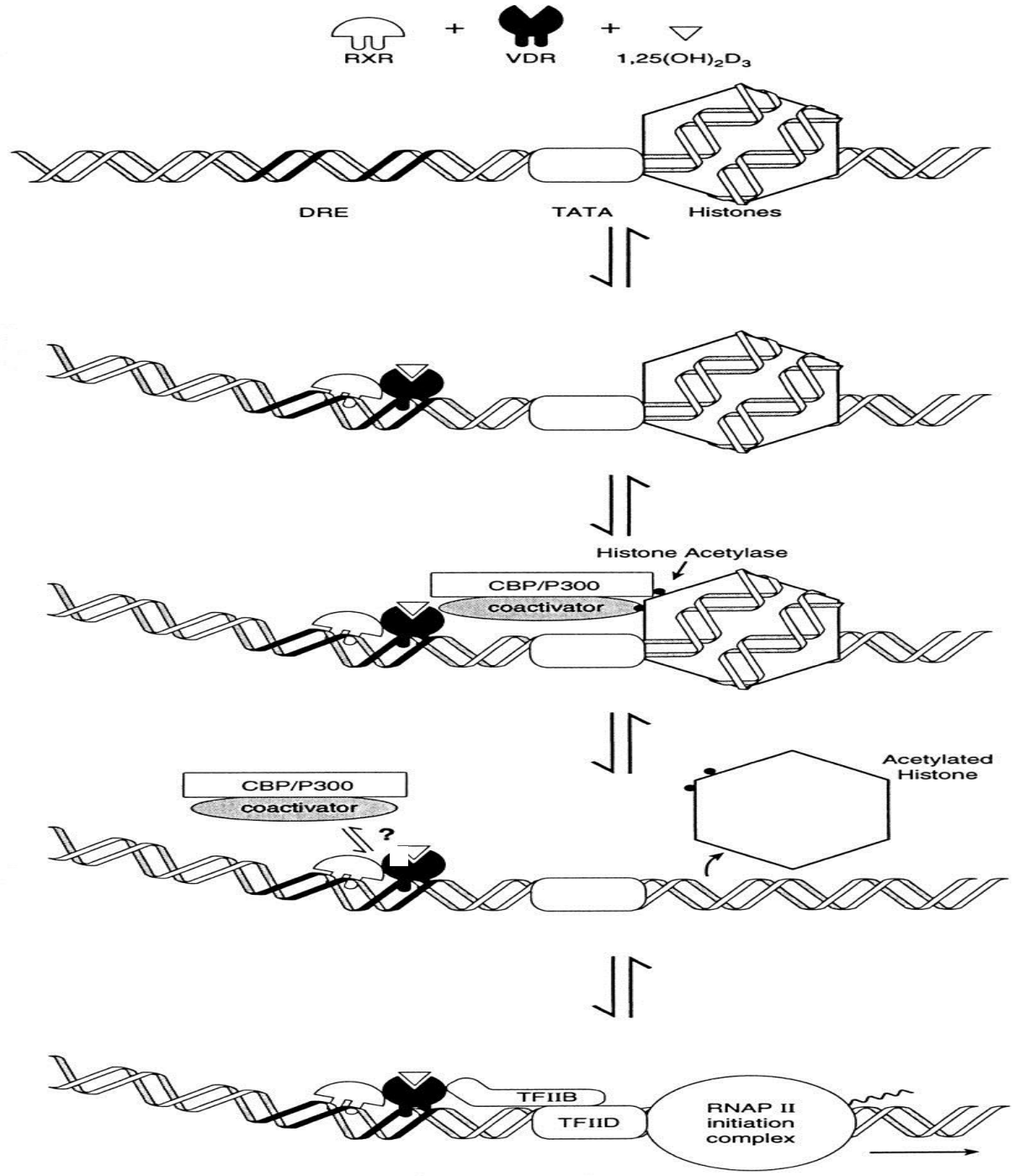


Figure 1-13: Example of binding of coactivator proteins to the heterodimer-DNA complex leads to histone acetylation and subsequent release of histones from DNA. Protein-protein interaction with transcriptional factor IIB (TFIIB) initiates transcription of target gene. Adapted from (Jones *et al*. 1998). CBP: Calcium-binding protein; VDRE: Vitamin D response elements; P300: transcriptional integrator p300; RNAP II: RNA polymerase II; TATA: Thymine Adenine Thymine Adenine; TFIID: Transcription factor IID

Retinoid X Receptor (RXR) as an obligate heterodimer partner for VDR

RXR is a member of the steroid superfamily of nuclear receptors (Dawson and Xia 2012). Its main functions are in modulating cell differentiation, metabolism and cell death (Dawson and Xia 2012). 9-cis retinoic acid (RA) is the natural ligand for RXR. The RXR exerts its biological functions almost exclusively by forming heterodimers with other receptors such as the thyroid hormone receptor, retinoic acid receptor (RAR) and vitamin D receptor (Bugge *et al*. 1992). In the vitamin D signalling pathway, it generally acts as a silent (non-liganded) partner that serves as an auxiliary factor to stabilise DNA binding of liganded-VDR to the VDRE (Bugge *et al*. 1992; Shaffer and Gewirth 2002). Upon binding to $1,25(OH)_2D_3$, the VDR-RXR heterodimers becomes relatively resistant to dissociation and diversion to other RXR pathways (Figure 1-14) (Torma *et al*. 1996; Thompson *et al*. 1998; Zhang *et al*. 2011). 9-cis RA binding to RXR causes release of nuclear corepressor such as NCoR, which further enhances transcriptional activity of VDR (Kang et al. 1997; Sanchez-Martinez et al. 2008).

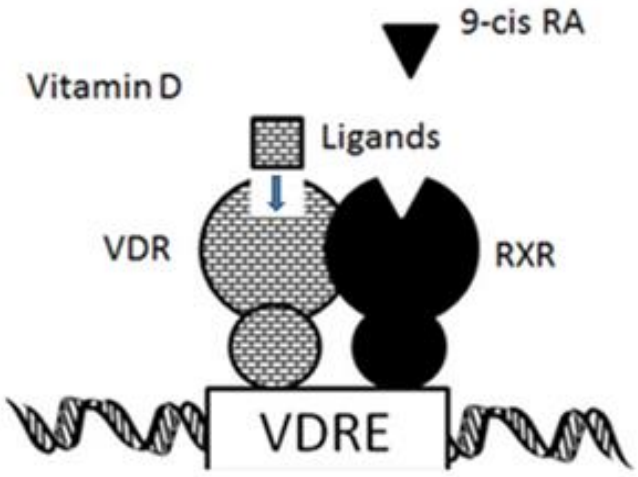


Figure 1-14: Schematic depiction of the VDR response to $1,25(OH)_2D_3$ binding. 9-cis retinoid acid (9-cis RA) is a natural ligand for retinoid X receptor (RXR). RXR stabilises the VDR-RXR heterodimers binding to the VDRE. Adapted from (Matsuda and Kitagishi 2013)

The precursors of $1,25(OH)_2D_3$ and their involvement in VDR-mediated signalling pathway

Cholecalciferol and $25(OH)D_3$ are the two precursors in the synthesis of $1,25(OH)_2D_3$. Cholecalciferol does not bind to vitamin D receptor. Hence, it does not exert any genomic effects (Ohyama and Shinki 2016). However, cholecalciferol can be metabolised by CYP11A1 to $20(OH)D_3$ and $20,23(OH)_2D_3$, both of which are partial agonists for the VDR (Figure 1-11) (Slominski et al. 2014). On the other hand, it has been suggested in the literature that $25(OH)D_3$ can control the expression of various genes and suppress cell proliferation via genomic action mediated by VDR (Ritter *et al*. 2006; Peng *et al*. 2009; Munetsuna *et al*. 2014). However, the affinity of $25(OH)D_3$ for VDR is remarkably low, approximately 100 times lower than that of $1,25(OH)_2D_3$ (Lips 2007).

1.5.2 Subcellular distribution of vitamin D receptors

At basal levels, *de novo* synthesised vitamin D receptors mainly reside in the cytoplasm (Prüfer *et al*. 1999; Ruth Wu-Wong *et al*. 2006). Cytoplasmic-nuclear cycling of the VDR is regulated largely by its binding to $1,25(OH)_2D_3$ (Racz and Barsony 1999). Dimerization of ligand-activated VDR with RXR promotes nuclear translocation and sub-nuclear targeting of VDRs (Prüfer et al. 2000; Yasmin et al. 2005). It has been proposed that subcellular distribution/localization of VDR also plays a role in regulating transcriptional activities of nuclear proteins (Prüfer and Barsony 2002). Nuclear import of VDR is mediated by importin α through binding of its nuclear localization sequences (NLS) (Prüfer and Barsony 2002). Excessive nuclear accumulation of VDR is prevented by export of VDR from the nucleus, a process mediated by nuclear export receptors that bind to specific nuclear export signal sequences (NES) (Prüfer and Barsony 2002). It has been suggested that calreticulin, a calcium-binding protein, may also serve as an export adapter through binding to DBP (Barsony *et al*. 1997; Prüfer and Barsony 2002). Lastly, microtubules play an important part in the intracellular traffic of vitamin D receptor (Akner *et al*. 1995). Sensitivity of fibrillary cytoplasmic VDR immunostaining pattern to

microtubule-disrupting drugs provides evidence to the vital role of microtubules in VDR nuclear translocation (Barsony and McKoy 1992; Barsony *et al*. 1997).

1.5.3 Non-genomic actions of $1,25(OH)_2D_3$

$1,25(OH)_2D_3$ exert its functions through both non-genomic and genomic pathways (Vuolo *et al*. 2012). In recent years, there have been an increasing number of studies reporting rapid activation of signal transduction pathways that do not involve nuclear gene transcription and protein synthesis following $1,25(OH)_2D_3$ treatment (Sitrin et al. 1999; Farach-Carson and Nemere 2003; Fleet 2004) (Figure 1-15). The non-genomic effects of $1,25(OH)_2D_3$ are mediated by binding to specific receptors on the plasma membrane of target cells that are coupled to activation of signalling molecules (Farach-Carson and Nemere 2003). These binding sites are referred to collectively as Membrane Associated, Rapid Response Steroid (MARRS) binding receptors (Farach-Carson and Nemere 2003; Nemere *et al*. 2004; Rohe *et al*. 2005; Norman 2006). Once bound, the $1,25(OH)_2D_3$-MARRS complex stimulates a variety of signalling pathways, through direct interaction with G-proteins or non-receptor tyrosine kinases such as Src kinase (Fleet 2004). The downstream consequences of these signal transduction pathways include opening of ion channels (calcium and chloride channels), activation of protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) (Fleet 2004; Norman 2006; Khanal et al. 2008; Nemere et al. 2010) (Figure 1-16). For example, it has been shown in avian myoblasts and murine colonic cells that $1,25(OH)_2D_3$ is capable of activating the G protein/c-src/PKCα pathway resulting in a rise of inositol triphosphate 3 (IP3), a secondary messenger that modulates diacylglycerol levels (Morelli et al. 1993; Sitrin et al. 1999). The rapid response to $1,25(OH)_2D_3$ was noted within seconds to minutes with an increase in calcium fluxes through voltage-operated channels (Morelli *et al*. 1993; Rohe *et al*. 2005). In another study using prostate cancer LNCaP cells, $1,25(OH)_2D_3$ bound to MARRS was shown to activate the JNK/SAPK MAPK signalling pathway, resulting in decreased cell invasiveness, which was observed within 10 minutes of treatment (Larsson *et al*. 2008). Studies by Schwartz *et al*. (2002) in matrix vesicles of growth zone murine chondrocytes which did not contain the nuclear VDR, found that $1,25(OH)_2D_3$ activates PKC through a G-protein,

resulting in modulation of MAPK activity that became evident within 9 minutes of treatment (Schwartz *et al*. 2002). There is no documented non-genomic activities of cholecalciferol and 25(OH)D_3 in vitamin D_3 signalling pathway.

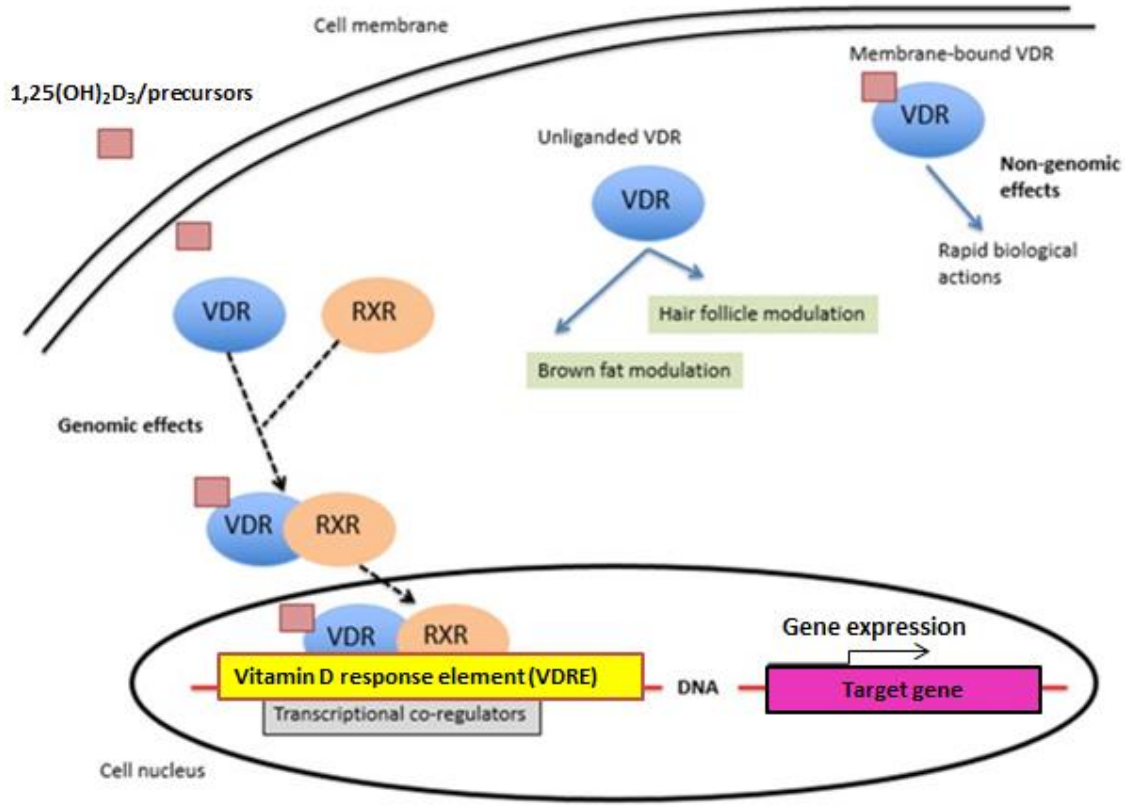


Figure 1-15: The non-genomic and genomic pathways of 1,25(OH)$_2$D$_3$. In the non-genomic pathways, 1,25(OH)$_2$D$_3$ bind to a subset of membrane-bound VDRs to trigger rapid, non-genomic actions. In classical genomic pathways, 1,25(OH)$_2$D$_3$ bind to VDR, resulting in a conformational change of VDR that facilitates binding with RXR. The ligand-VDR-RXR complex then translocates into the nucleus and binds to vitamin D response elements (VDRE) within the DNA that regulate gene transcription. Genomic pathway is regulated by transcriptional co-regulators, such as transcriptional factor II (TFII). Adapted from (Elangovan *et al*. 2017)

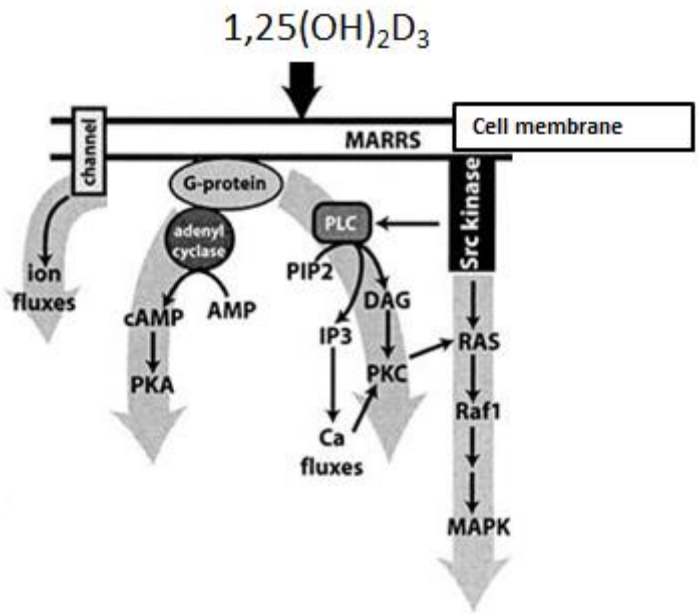


Figure 1-16: The non-genomic pathways of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ binds to MARRS receptors and activates signalling molecules such as G-protein and Src kinase. The downstream effects of this are opening of ion channels (e.g. calcium and chloride channels) and activation of secondary messengers such as PKA, PKC and MAPK that regulates physiological responses. Adapted from (Fleet 2004)

1.6 Current understanding on the autocrine/paracrine roles of vitamin D in skin and hair follicles

The evolution of light pigmentation at high latitudes suggests the importance of vitamin D_3 photosynthesis in the skin (Jablonski and Chaplin 2010). It is now recognised that vitamin D indeed, plays a significant role in skin health. The skin is capable of autocrine production of $1,25(OH)_2D_3$ (Milde *et al*. 1991; Bikle 2011) and is also an important target tissue (Milde *et al*. 1991).

Skin cells differentiation and proliferation

Vitamin D_3 analogues are commonly used to treat skin conditions such as psoriasis vulgaris (Darley *et al*. 1996; Norris 2005). Their beneficial effects were initially attributed to anti-proliferative and differentiation-inducing effects on epidermal keratinocytes (Hansen *et al*. 1996). $1,25(OH)_2D_3$ induces differentiation and growth inhibition by causing cell cycle arrest at the G1/G0 phase in synchronized normal

human keratinocytes (Kobayashi *et al*. 1998). In the presence of low concentrations of $1,25(OH)_2D_3$ (0.12-12nM), basal cell populations within the epidermis are stimulated to differentiate into squamous cells (Hosoi *et al*. 1985). The effects of vitamin D analogues on epidermal cell proliferation and differentiation are dose-dependent, promoting proliferation of keratinocytes at low concentration and inhibiting proliferation at higher pharmacological doses ($>10^{-8}$ M) (Gniadecki 1996). Loss of VDR function or absence of $1,25(OH)_2D_3$ disrupts differentiation of the epidermis and results in hyperproliferation of the basal keratinocytes (Bikle 2011).

Photoprotective effect and melanogenesis

Ultraviolet (UV) radiation increases nitric oxide products generated by skin cells (Bruch-Gerharz *et al*. 1998). The photo-protective effect of $1,25(OH)_2D_3$ is attributed to its antioxidant properties (Hanada *et al*. 1995; Lee and Youn 1998). In murine studies, keratinocytes exposed to $1,25(OH)_2D$ showed increased tolerance (higher cell survival) to ultraviolet B (UVB) injury (Hanada *et al*. 1995). Inactivation of the VDR enhances UV-induced skin tumorigenesis in mice (Ellison *et al*. 2008). In human keratinocytes, $1,25(OH)_2D_3$ treatment was shown to increase p53 (tumour suppression gene) expression and decreases nitric oxide products *in vitro* (Gupta *et al*. 2007).

Melanin synthesis by murine melanoma cells was reportedly stimulated by $1,25(OH)_2D_3$ *in vitro* (Oikawa and Nakayasu 1974; Hosoi *et al*. 1985). In primary human melanocytes *in vitro*, vitamin D increases melanogenesis and the tyrosinase content of melanocytes via its anti-apoptotic effect (Mason 2000; De Haes *et al*. 2003). Using an immature melanocyte cell line derived from mouse neural crest cells, it has been shown that $1,25(OH)_2D_3$ acts to induce immature melanocytes in the bulge region of the hair follicle to produce melanin by stimulating their differentiation *in vitro* (Watabe *et al*. 2002). Upregulation of pigment production by $1,25(OH)_2D_3$ confers a protective effect to the skin cells from DNA damage and tumorigenesis. This is highlighted by epidemiology studies that found that the risk of high stage melanoma was inversely proportional to serum vitamin D_3 level (Nurnberg *et al*. 2009; Gambichler *et al*. 2013).

Immunomodulatory actions

Various immune cell types such as lymphocytes, macrophages, neutrophils and dendritic cells express VDR and possess CYP27B1 (1α-hydroxylase) for autocrine production of the active vitamin D metabolites (Amento 1987; Aranow 2011; Di Rosa *et al*. 2011). The human cathelicidin antimicrobial peptide (CAMP) gene is now known to be a direct target of the VDR (Gombart *et al*. 2005). $1,25(OH)_2D_3$ regulates the antimicrobial peptide cathelicidin, enhances Toll-like receptor 2 (TLR2) functions and improves dermatitis and wound healing (Schauber *et al*. 2007). It has also been shown clinically that administration of oral vitamin D induces cathelicidin production in patients with atopic dermatitis (Hata *et al*. 2008). There is also a complex interplay between $1,25(OH)_2D_3$ and the skin microbiome. In murine models *in vivo*, it has been shown that $1,25(OH)_2D_3$ may be able to suppress tissue inflammation by altering the microbiome at the airway lining and colon (Lagishetty *et al*. 2010; Gorman *et al*. 2013). There is currently no data on the microbiome altering properties of $1,25(OH)_2D_3$ in cutaneous wounds.

Hair follicle cycling, development and growth

The hair follicle is a highly hormone-sensitive mini organ (Reichrath et al. 1994). In the murine model, the presence of both the VDR and $1,25(OH)_2D_3$ is essential for hair follicle cycling (Sakai and Demay 2000; Chen *et al*. 2001; Sakai *et al*. 2001). It has now been established that VDR is not only expressed in epidermal keratinocytes, but also in dermal papilla cells and outer root sheath cells of the hair follicles (Reichrath et al. 1994; Kong et al. 2002; Cianferotti et al. 2007). Murine studies have shown that VDR expression is related to the hair cycle, expression is increased in dermal papilla cells and outer root sheath keratinocytes during anagen IV-VI and in catagen, and decreased during telogen and anagen I-III (Reichrath et al. 1994). These two sites correspond to the location of mesenchymal and epithelial stem cells found in hair follicles normally, suggesting its importance in both hair follicle and interfollicular epidermal regeneration (Yang and Cotsarelis 2010; Mistriotis and Andreadis 2013). Similarly, murine studies have shown that the VDR is

required for the initiation of the postnatal hair follicle cycle, and knockout of VDR resulted in alopecia (Kong et al. 2002). Furthermore, the matrix cells of hair follicles express all the necessary enzymes for synthesising $1,25(OH)_2D_3$, suggesting they play an important autocrine and paracrine roles in regulating $1,25(OH)_2D_3$ homeostasis (Zehnder *et al*. 2001). In fact, absence of $1\alpha,25(OH)_2D_3$, the ligand for VDR, results in hair loss in mice (Chen *et al*. 2001; Meindl *et al*. 2005; Teichert *et al*. 2010). This effect is VDR-independent, further supporting the notion that the hair follicle is an important target cell for 1, $25(OH)_2D_3$. Mutations in the VDR gene are associated with alopecia in humans and topical $1,25(OH)_2D_3$ administration prevents chemotherapy-induced alopecia in mice (Takeda *et al*. 1987; Jimenez and Yunis 1992). Clinically, there have been case reports of the successful treatment of alopecia areata with calcipotriol (a vitamin D analogue), suggesting a possible role of vitamin D in restoration of hair follicle dysfunction (Kim *et al*. 2012).

1.6.1 Vitamin D signalling in response to wounding

Upon injury, cytokines and growth factors are released at the wound site (Bikle *et al*. 2014). These stimulate proliferation and migration of dermal and epidermal cells for wound closure (Bikle *et al*. 2014). Calcium interacts with $1,25(OH)_2D_3$-VDR pathways in regulating skin differentiation and proliferation processes (Bikle *et al*. 2014; Mostafa and Hegazy 2015). This is achieved by induction of the calcium receptor and phospholipase C via $1,25(OH)_2D_3$ binding in human keratinocytes (Pillai *et al*. 1995). Expression of involucrin, transglutaminase, loricrin, and filaggrin are upregulated, thereby enhancing keratinocyte differentiation into the cornified envelope, and eventually leading to reformation of the epidermal barrier (Mostafa and Hegazy 2015).

Vitamin D and calcium signalling are equally necessary for an adequate immunomodulatory response to epidermal wounding (Bikle *et al*. 2014). $1,25(OH)_2D_3$-dependent actions of the VDR are fundamental in promoting a normal inflammatory response to cutaneous injury (Luderer *et al*. 2013). In murine studies, absence of VDR has been shown to result in defective granulation tissue formation, which was attributed to impaired TGF-β signalling and a decrease in macrophage

recruitment to the wound site (Luderer *et al*. 2013). In a separate study, mice lacking the VDR or CYP27B1 showed a defective innate immune response, downregulated β-catenin transcription, reduced proliferation of keratinocytes at the wound edges and delayed wound healing (Oda *et al*. 2015). Lastly, vitamin D plays a vital role in maintaining the hair follicle stem cell population, which is vital for good quality wound repair (Chen *et al*. 2001; Cianferotti *et al*. 2007; Teichert *et al*. 2010; Demay 2012).

1.6.2 The role of dermal fibroblasts (DFs) in $1,25(OH)_2D_3$ production and its function as a target cells

Surprising, there are limited studies investigating the role of human dermal fibroblasts in $1,25(OH)_2D_3$ production and their potential as target cells in cutaneous wound healing. Scatchard analysis using human dermal fibroblasts showed that cultured fibroblasts grown from infant foreskin expressed the VDR (Feldman *et al*. 1980). A Kd for $[^3H]1,25(OH)_2D_3$ of 0.2nM and a Bmax of approximately 40 fmol/mg cytosol protein was reported in this study (Feldman *et al*. 1980). Since then, dermal fibroblasts have been used to demonstrate VDR mutations in patients with hereditary vitamin D-resistant rickets (HVDRR) (Feldman and Malloy 2014). A correlation with the development of alopecia in this group of patients was also established (Feldman and Malloy 2014). However, little is known about the role of dermal fibroblasts in vitamin D homeostasis locally. Using a Northern Blot technique, researchers have shown that dermal fibroblasts derived from human foreskin respond to $1,25(OH)_2D_3$ by upregulating CYP24A1 mRNA expression (Vantieghem 2006). These dermal fibroblasts also expressed VDR, CYP2R1 and CYP24A1 mRNA. However, CYP27B1 (1α-hydroxylase) RNA was not detected by Northern Blot analysis (Vantieghem 2006). In a recent study utilising real time PCR and ELISA techniques, Norlin *et al* showed that primary human dermal fibroblasts derived from skin biopsies expressed all the vitamin D_3 metabolising enzymes and are thus potentially capable of autocrine production of active $1,25(OH)_2D_3$ (Norlin et al. 2017). However, the source of the skin biopsies (i.e. the sites, hairy/non-hairy, and age groups of patients) could not be identified, which presents a limitation to this study (Norlin et al. 2017).

1.7 Prevalence of Vitamin D deficiency in the general population

Over the past decades, vitamin D deficiency as an epidemic has been recognised (Blair 2012). It has been linked with a multitude of diseases, including diabetes, tuberculosis, pancreatic cancer, breast cancer, bone diseases, and cardiovascular diseases (Autier *et al*. 2014). The importance of vitamin D in general health was reaffirmed and highlighted by the establishment of an European Union (EU) funded project that aims to provide high quality scientific evidence in the prevention of vitamin D deficiency among EU citizens (Cashman *et al*. 2016). The four-year "Optimal Vitamin D in Nutrition" (ODIN) project that started in 2013 has identified a 13% prevalence of vitamin D deficiency in all EU population across a variety of ages (Cashman *et al*. 2016). In the United Kingdom (UK), pilot studies and regional monitoring suggests that vitamin D deficiency is likely to affect at least half the UK's white population, up to 90% of the multi-ethnic population and a quarter of all children living in Britain (Blair 2012).

Surprisingly, the population that are at risk of developing chronic wounds are often found to be vitamin D deficient. A study looking at differences in the capacity for synthesis of vitamin D_3 in human skin among different age groups reported an chronological age-dependent decrease in the epidermal concentrations of provitamin D_3 (7-dehydrocholesterol) in surgically-obtained human skin (MacLaughlin and Holick 1985). This study also showed an age-dependent reduction in the capacity of the skin to produce cholecalciferol (MacLaughlin and Holick 1985). Vitamin D deficiency is also more prevalent among diabetic patients (Hyppönen *et al*. 2001; Mohr *et al*. 2008). Clinical trials have shown that oral vitamin D supplementation results in better venous ulcer healing (Burkiewicz et al. 2012). Unfortunately, data from molecular biology studies investigating the effect of vitamin D deficiency on wound healing is still limited (Molnar *et al*. 2014).

1.8 Aim and objectives

While human epidermal keratinocytes are the main source of vitamin D, they are also important target cells. However, little is known about the role of dermal fibroblasts in the autocrine and paracrine regulation of biologically active vitamin D. The main aim of this project was to establish the effects of vitamin D on human dermal fibroblasts, in particular investigating its potential role in cutaneous wound healing.

The first aim of this study was to investigate the autocrine and paracrine roles of primary human dermal fibroblasts in $1,25(OH)_2D_3$ homeostasis *in vitro* at basal conditions. The mRNA expression of CYPs by dermal fibroblasts was quantified and compared with both donor-matched and non-matched epidermal keratinocytes. VDR, which is an important mediator of genomic actions by $1,25(OH)_2D_3$, was also quantified and its relative expression in dermal fibroblasts and keratinocytes established.

There is evidence in the literature suggesting $1,25(OH)_2D_3$ upregulates the migration of epidermal keratinocytes *in vitro*. However, studies looking at the effect of vitamin D_3 on dermal fibroblast migration after wounding are lacking. This study aims to compare the effects of $1,25(OH)_2D_3$ and its precursors on migration of primary cultures of epidermal keratinocytes and dermal fibroblasts; effects on fibroblasts were also determined under a variety of conditions (i.e. different treatment concentrations, with or without serum). Furthermore, to investigate whether the modulatory effect on migration was mediated by VDR, knockdown of VDR was also performed.

The next aim of this study was to determine how $1,25(OH)_2D_3$ and its precursors modulate VDR and CYP expression in primary cultures of epidermal keratinocytes and dermal fibroblasts at basal conditions. Subsequently, their transcriptional expression was also determined under wounded conditions. The genomic pathway

in which $1,25(OH)_2D_3$ or its precursors exert their biological functions was investigated by measuring VDR protein expression.

Dermal fibroblasts are heavily involved in wound contraction, extracellular matrix deposition and degradation, and the modulation of collagen content during wound healing. The effects of $1,25(OH)_2D_3$ and its precursors on these aspects of wound healing were assessed by measuring metabolic activity, expression of markers for myofibroblast differentiation, secretion of matrix metalloproteinases and changes in the ratio of type I and III collagen in primary cultures of dermal fibroblasts.

Finally, an *ex vivo* human skin wound healing model was developed that more closely resembles the actual three-dimensional microenvironment in human wounds to establish the impact of $1,25(OH)_2D_3$ and its precursors on wound closure.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The following chemicals were supplied by

Sigma-Aldrich, UK:

- 1α,25-Dihydroxyvitamin D3
- 25-hydroxyvitamin D3
- 2-Mercapto-ethanol
- Acrylamide
- Cholecalciferol
- Dimethyl sulfoxide **(**DMSO)
- Eosin Y-solution 0.5% alcoholic
- Ethanol
- Formaldehyde
- Gelatin
- Mayer's Haematoxylin MHS-16
- Phosphate buffered saline
- Thermo Scientific Pierce Tetramethylethylenediamine (TEMED)
- Triton X-405

Thermo Fisher Scientific, UK:

- Alamar Blue®
- Lipofectamine® RNAiMAX transfection reagent
- HistoMount

Abcam, UK:

- Phorbol 12-myristate 13-acetate (PMA)

2.1.2 Human Skin samples

Skin samples from healthy patients undergoing facelift plastic surgery, abdominoplasty or breast reduction surgery were obtained from female donors with informed consent. The samples were collected by Ethical Tissue Bank from the hospital and immediately placed in transporting media (Section 2.1.3.1) until it was processed no longer than 6 h later.

2.1.3 Cell Culture

2.1.3.1 Transporting media

Minimum Essential Medium (MEM), GlutaMAX supplement was supplemented with 10% foetal calf serum (FCS), 250 Units/ml penicillin and 250 µg/ml streptomycin (Pen/Strep), 7.5 µg/ml of amphotericin B and 6.15 µg/ml of sodium deoxycholate, both contained in fungizone antimycotic and 2 mM of L-alanyl-L-glutamine dipeptide in 0.0085% of NaCl or glutaMAX and stored at 4˚C. (All reagents/chemicals were from GIBCO, UK)

2.1.3.2 Primary Keratinocyte Growth Media (PKGM)

Primary keratinocyte growth media (PKGM) was made up using keratinocyte growth media 2 (PromoCell, UK) supplemented with 0.004 ml/ml of bovine pituitary extract, 0.125 ng/ml of epidermal growth factor (recombinant human), 5 µg/ml of insulin (recombinant human), 0.33 µg/ml of hydrocortisone, 0.39 µg/ml of epinephrine, 10 µg/ml of tranferrine, holo (human), 100 Units/ml penicillin with 100 µg/ml streptomycin (GIBCO) and 2.48 µg/ml fungizone (GIBCO). Keratinocyte growth media 2 was stored at 4 ˚C and all supplements at -20 ˚C. PKGM was kept at 4 ˚C and used within 4 weeks

2.1.3.3 Primary Keratinocyte Starvation Media (PKSM)

Primary keratinocyte starvation media (PKSM) was made up using keratinocyte growth media 2 (PromoCell, UK) and all the supplements as in PKGM apart from the

epidermal growth factor and fungizone. PKSM was kept at 4 ˚C and used within 4 weeks.

2.1.3.4 Primary Fibroblast Growth Media (PFGM)

Primary fibroblast growth media (PFGM) was made up using Dulbecco's modified eagle medium (DMEM) with L-glutamine (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum (FBS) (GIBCO), 100 Units/ml penicillin and 100 µg/ml streptomycin (Pen/Strep) (GIBCO), 2.5 µg/ml of amphotericin B and 2.05 µg/ml of sodium deoxycholate, both contained in fungizone antimycotic (GIBCO). DMEM was stored at 4 ˚C and all supplements at -20 ˚C. Once PFGM was prepared, it was kept at 4 ˚C and used within four weeks.

2.1.3.5 Primary Fibroblast Starvation Media (PFSM)

Primary Fibroblast Starvation Media (PFSM) was made with phenol red-free Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine (Sigma-Aldrich, UK) supplemented with 100 Units/ml penicillin and 100 µg/ml streptomycin (Pen/Strep) (GIBCO) and 2.5 µg/ml of amphotericin B and 2.05 µg/ml of sodium deoxycholate, both contained in fungizone antimycotic (GIBCO). Once PFSM was prepared it was stored at 4 ˚C and used within four weeks.

2.1.3.6 Primary Cells Freezing Media

Cryo-SFM (Promocell) was used to freeze primary human keratinocytes. Dermal fibroblasts were frozen in media consisting of 80% of PFGM (see Section 2.1.2.4), 10% FBS (GIBCO) and 10% dimethyl sulfoxide **(**DMSO).

2.1.4 Human primary cell lines used

A list of human primary cell lines that were established for this study is given in table 2-1. Each cell line was assigned a sample identification code (bottom row) for simplicity in methods and results description.

	Primary epidermal keratinocytes					Primary dermal fibroblasts										
Sex	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
Age	51	55	58	59	60	36	38	53	55	56	57	59	59	59	60	66
Passage	2	3	3	3	3	3	3-5	4	3	3	3	3	3-5	4	3	3
Sample code	EK1	EK2	EK3	EK4	EK5	DF1	DF2	DF3	DF4	DF5	DF6	DF7	DF8	DF9	DF10	DF11

Table 2-1: All primary cell lines used in the experiments were derived from female (F) facial skin. Donor-matched epidermal keratinocytes and dermal fibroblasts are highlighted in purple: EK2 and DF4; EK4 and DF7; EK5 and DF10. EK denotes epidermal keratinocyte and DF denotes dermal fibroblast.

2.2 Methods

2.2.1 Establishment of primary cells cultures from human skin

The skin samples were all derived from female facelift surgery. They arrived at the laboratory in transport media as detailed in section 2.1.2.1 and processed within 12 hours. Each skin sample was transferred to a 60mm tissue culture dish (Starstedt) and washed with PBS three times. Fat tissue was carefully dissected away. The remaining skin was cut into squares of tissue measuring approximately 1 to 1.5cm^2. They were then placed in 5.2 U/ml of dispase (GIBCO) in PBS with 100 Units/ml penicillin and 100 µg/ml streptomycin (GIBCO) and 2.5 µg/ml of amphotericin B and 2.05 µg/ml of sodium deoxycholate (Fungizone antimycotic, GIBCO), and kept at 4ºC overnight.

With the aid of a Leica dissecting microscope, the epidermis was carefully separated from the underlying papillary dermis using a pair of fine-tipped, non-tooth forceps. The epidermis collected was placed in a 15ml Falcon tube containing

primary keratinocyte growth media (PKGM) whereas the remaining dermis was placed in primary fibroblast growth media (PFGM).

2.2.1.1 Primary epidermal keratinocyte isolation, culture, and passaging

The epidermis was transferred to a separate 15ml Falcon tube containing 3 ml of 0.05% Trypsin/EDTA (Sigma-Aldrich, UK) and incubated at 37ºC for 5 minutes. The Falcon tube containing the epidermis was then vortexed briefly for 10 to 15 seconds. After removing the excess epidermis, 3 ml of trypsin neutralising solution (TNS) (Lonza, UK) was added. The suspension was centrifuged at 800 rpm for 5 minutes. The resulting pellet was re-suspended in PKGM (section 2.1.2.2). A cell count was performed using a Neubauer haemocytometer (Hawksley, UK). The cells were then cultured in a T75 flask (Corning, UK) containing 15 ml of PKGM (section 2.1.2.2) at a cell density of 30,000 cells/ml. The T75 flasks were transferred to a Heracell^{TM} 150i humidified incubator and incubated at 37ºC and 5% CO_2 in air. The PKGM (Section 2.1.2.2) was changed every other day until the primary epidermal keratinocytes reached 80% confluency. This normally took between 7 to 14 days.

Once they had reached 80% confluency, the primary epidermal keratinocytes were passaged. They were washed with PBS twice before 0.5 mM (2.5 ml) of ethylenediaminetetraacetic acid (EDTA) (GIBCO, UK) was added. The cells were incubated at 37ºC for 4 minutes to disrupt cell-cell interactions before the EDTA was discarded. Subsequently, 0.05% (2 ml) Trypsin/EDTA (Sigma-Aldrich, UK) was added and the keratinocytes were incubated for another 2 minutes at 37ºC. The T75 flasks were examined under a phase contrast microscope to check that cells had detached from the flasks. Trypsin Neutralising Solution (TNS) (Lonza, UK) that was double in volume to the Trypsin/EDTA solution (4 ml) was added to each flask and the keratinocytes resuspended in the TNS.

The cell suspension was transferred to a 15 ml Falcon tube (Starstedt, UK). A cell count and viability count was determined using a Neubauer haemocytometer (Hawksley, UK). To determine cell viability, trypan blue solution 0.4% (Sigma-Aldrich, UK) was used. The following formulas were used to determine total number of viable and non-viable cells:

Total number of cells/ml = total cells counted x (dilution factor/number of squares) x 10^4

% Viability = Total number of viable cells/total number of cells x 100

Once the cell count was determined, the cell suspension was centrifuged for 5 minutes at 1000 rpm. The supernatant was removed and the cell pellet re-suspended in PKGM (Section 2.1.2.2). For routine keratinocyte passaging, the cells were split at a ratio of 1:2 and cultured in T25 or T75 flasks.

2.2.1.2 Primary dermal fibroblast isolation, culture and passaging

Between 6 and 10 pieces of dermis measuring approximately $1cm^2$ were placed into a T75 flask (Corning, UK) with the papillary dermis facing down. Five millilitres of PFGM was added to each flask and they were transferred to the Heracell^{TM} 150i incubator and cultured at 37ºC and 5% CO_2 in air. The dermal explants were checked the following day to make sure that they were attached to the flasks. The PFGM (Section 2.1.2.4) was changed every other day until the primary dermal fibroblasts reached 80% confluency. This normally took between 10 to 21 days.

The dermal fibroblasts were passaged once they reached 80% confluency. The monolayers were washed twice with PBS before0.05% (2 ml) of Trypsin/EDTA (Sigma-Aldrich, UK) was added and the cells incubated at 37ºC for 5 minutes. Cell detachment from the flask was determined under the phase contrast microscope. PFGM containing 10% FBS (Section 2.1.2.4) that was double in volume to the Trypsin/EDTA solution (4 ml) was added to each flask. The cell suspension was transferred to a 15 ml Falcon tube. Cell count and viability was determined as described previously (Section 2.2.1.1). Once the cell count was determined, the cell suspension was centrifuged for 5 minutes at 1000 rpm. The supernatant was removed and the cell pellet re-suspended in 1mL of PFGM (Section 2.1.2.4). For routine passaging, dermal fibroblasts were split with a ratio of 1:3 and cultured in T25 or T75 flasks.

2.2.2 Cryopreserving and thawing of human epidermal keratinocytes and dermal fibroblasts

Both cell types can be frozen using the appropriate cryopreservation medium. Once the cells were processed and centrifuged (Section 2.2.1.1 and Section 2.2.1.2) and the supernatant discarded, the cells were re-suspended in 1ml of cryopreservation medium; Cryo-SFM for epidermal keratinocytes and fibroblasts freezing medium for dermal fibroblasts (Section 2.1.2.5).

The cell suspension was transferred to 1ml CryoPure tubes (Sarstedt). Sample tracking number, passage number, gender and age of the patient, date of freezing and size of flask were recorded on the CryoPure tubes label as appropriate. The tubes were placed in liquid nitrogen vapour tank for 24 hours before finally being transferred to the liquid nitrogen storage tank.

Thawing of cells was done rapidly (<1 minute) by holding the CryoPure tube under warm running tap water. The thawed cell suspension was added to pre-warmed growth medium (37ºC) in T25 or T75 flasks. The media was changed after 24 hours once the cells were attached.

2.3 Quantitative real-time PCR (qRT-PCR) to determine transcriptional expression of genes

2.3.1 Relative mRNA expression of VDR, CYP2R1, CYP27B1, and CYP24A1 in human epidermal keratinocytes (EK) and dermal fibroblasts (DF) at basal level.

Primary epidermal keratinocytes and dermal fibroblasts were plated in 6-well plates (Greiner Bio-one, UK) at a density of 50,000 cells per well and 30,000 cells per well respectively in two mls of PKGM or PFGM (Section 2.1.2) The medium was changed every other day. When cells were 80-90% confluent, they were ready for mechanical wounding.

For donor-matched epidermal keratinocytes and dermal fibroblasts (Table 2-2), two horizontal and two vertical scratch marks were created across the monolayer of cells using a 10µl pipette tip (0.2mm in width) as shown in Figure 2-1A. A template was used to ensure consistency in producing the scratch marks. After scratching, all the wells were washed with PBS twice to ensure removal of any dead or floating cells. The cells were then incubated in either PFGM or PKGM (Section 2.1.2) at 37ºC for a further 24 hours, before RNA was harvested for VDR, CYP2R1, CYP27B1, and CYP24A1 mRNA expression analysis.

For non-donor-matched keratinocytes and fibroblasts (Table 2-2), the RNA was harvested following a scratch-wound migration assay, therefore only one vertical scratch wound (0.8mm in width) was created using a scratching device provided by Dr. Ola Kamala using a predesigned template as shown in Figure 2-1B (Kamala 2014). After scratching, incubation and RNA extraction were performed the same way as described for the donor-matched samples.

	Epidermal keratinocytes					**Dermal fibroblasts**							
Sex	F	F	F	F	F	F	F	F	F	F	F	F	F
Age	51	55	58	59	60	53	55	56	57	59	59	59	60
Passage	2	3	3	3	3	4	3	3	3	3	5	4	3
Sample code	EK1	EK2	EK3	EK4	EK5	DF3	DF4	DF5	DF6	DF7	DF8	DF9	DF10

Table 2-2: Primary human epidermal keratinocytes and dermal fibroblasts used in qRT-PCR to determine relative expression of VDR, CYP2R1, CYP27B1 and CYP24A1 expression at basal level. Donor-matched epidermal keratinocytes and dermal fibroblasts are highlighted in purple: EK2 and DF4; EK4 and DF7; EK5 and DF10. EK denotes epidermal keratinocyte and DF denotes dermal fibroblast.

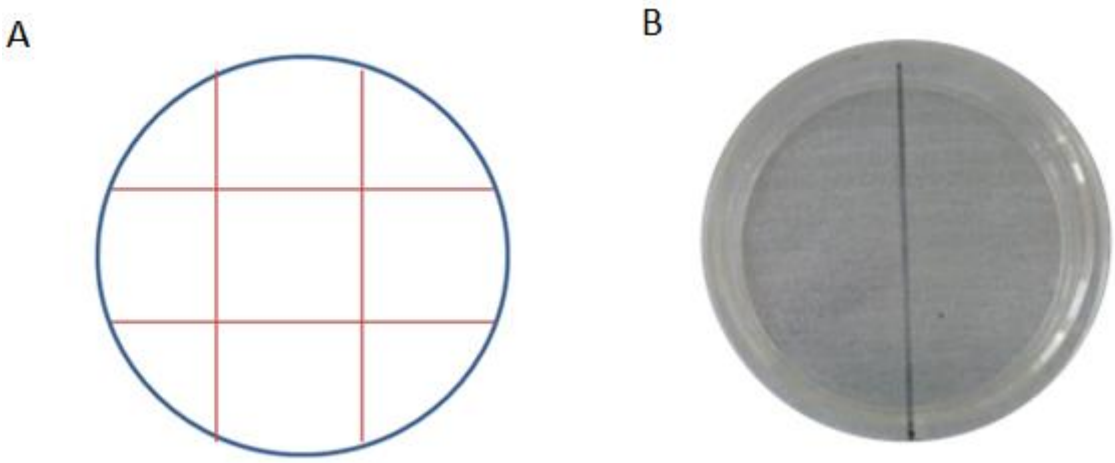


Figure 2-1: Scratch marks across monolayer of cells within a well in a 6-well plate for total RNA collection. A) Method used for donor-matched epidermal keratinocytes and dermal fibroblasts. Two horizontal and two vertical scratch marks were created across the monolayer of cells using a 10µl pipette tip (width of scratch - 0.2mm); B) Method for non-donor-matched epidermal keratinocytes and dermal fibroblasts. A single vertical scratch was created using a scratching device provided by Dr. Ola Kamala (width of scratch – 0.8mm).

2.3.2 Relative mRNA expression of VDR, CYP2R1, CYP27B1, and CYP24A1 in human epidermal keratinocytes (EK) and dermal fibroblasts (DF) after incubation with 1,25$(OH)_2D_3$ or its precursors.

Total RNA was harvested from epidermal keratinocytes or dermal fibroblasts following the assessment of migrationas described in Section 2.4.1. The age of the donors, passage number and sample code of epidermal keratinocytes and dermal fibroblasts used are specified in Table 2-3. The method used for scratching is as described in Section 2.3.1 and Figure 2-1B.

	Primary epidermal keratinocytes			Primary dermal fibroblasts		
Sex	F	F	F	F	F	F
Age	55	59	60	59	59	60
Passage	3	3	3	3	4	3
Sample code	EK2	EK4	EK5	DF7	DF8	DF10

Table 2-3: Primary human epidermal keratinocytes and dermal fibroblasts used in qRT-PCR to determine relative expression of VDR, CYP2R1, CYP27B1 and CYP24A1 expression in scratched assay after incubation with $1,25(OH)_2D_3$ or its precursors.

2.3.3 Relative mRNA expression of collagen I and III in human dermal fibroblasts (DF) after incubation with $1,25(OH)_2D_3$

Total RNA was harvested from dermal fibroblasts as cultured and treated as described in Section 2.4.1. The age of the donors, passage number and sample code of dermal fibroblasts used are specified in Table 2-4. Method used for scratching is as described in Section 2.3.1 and Figure 2-1B.

	Primary dermal fibroblasts		
Sex	F	F	F
Age	59	59	60
Passage	3	4	3
Sample code	DF7	DF8	DF10

Table 2-4: Primary dermal fibroblasts used in qRT-PCR to determine relative expression of collagen I and III expression in scratched assay after incubation with $1,25(OH)_2D_3$.

2.3.4 RNA extraction and quantification

Total RNA extraction was performed with the RNeasy Mini Kit (QIAGEN, UK). 10µL/ml of β-mercaptoethanol (Sigma-Aldrich, UK) was added to Buffer RLT provided with the kit. 200µL of the mixture was then added to the cell monolayer in

each well (in triplicate), and pipetted up and down several times. The resulting suspension was transferred to a 1.5 ml micro centrifuge tube. One volume of 70% ethanol was added to the cell lysate and mixed well by pipetting up and down briefly. Immediately after, the resulting suspension was transferred to a RNeasy spin column and centrifuged at 13000 rpm for 15 seconds at 4 ºC. Flow-through was discarded and 350µl of buffer RW1 (Table 2-5) was added to wash the spin column by centrifuging at 13000 rpm for 15 seconds at 4ºC. DNase digestion was performed using the RNase-free DNase set (QIAGEN 2012) to remove possible DNA contamination of the sample. 70µl of RNAse-free Buffer RDD (Table 2-5) was mixed with 10 µl (2.72 U/ µl) of DNase and the resulting mixture (80µl of 0.34 U/µl DNAse I) was added to the spin columns and allowed to stand for 15 minutes at room temperature. Following on-column DNA digestion, a further 350µl of buffer RW1 was added to each spin column and centrifuged at 13000 rpm for 15 seconds at 4 ºC. Five hundred microliter of Buffer RPE (Table 2-5) was used to wash each column with a centrifugation speed of 13000 rpm for 15 seconds at 4 ºC. The flow-through was discarded and the RPE buffer wash was repeated with a longer centrifugation period of 2 minutes, while maintaining the same speed and temperature. The RNAeasy column was placed in a new collection tube and further centrifuged at 13,000 rpm for 1 minute at 4 ºC for removal of residual RPE buffer. Lastly, the RNeasy spin columns were placed in 1.5 ml RNase free collection tubes and 25 to 30 µl of RNase-free water was added to each. They were centrifuged at 13,000 rpm for 1 minute at 4 ºC. The RNA collected in the tubes was then stored at -80ºC until future use.

NanoPhotometer (IMPLEN) was used for RNA quantification. One microliter of nuclease-free water was used as a blank for calibration. For quantification of RNA, absorbance measurements were made using the NanoDrop spectrophotometer. The purity of RNA extracted was determined by the ratio of absorbance at 260nm and 280nm given by the spectrophotometer reading. A ratio of ~2.0 is accepted as reasonably ‘pure’ RNA, indicating the sample is unlikely to be contaminated by the presence of protein, phenol or other contaminants. On the other hand, nucleic acid purity was determined using the 260/230 values. A range of 2.0-2.2 was considered to be acceptable.

Reagents	Details/composition of reagents
Buffer RW1	Contains a guanidine salt, as well as ethanol and is used as a stringent washing buffer that efficiently removes biomolecules such as carbohydrates, proteins, fatty acids etc., that are non-specifically bound to the silica membrane of the RNeasy mini spin columns (QIAGEN, UK)
Buffer RDD	Buffer RDD, when used with DNase from the RNAse-Free DNase Set (QIAGEN, UK), provides efficient on-column digestion of DNA and also ensures that the RNA remains bound to the RNeasy mini spin columns (QIAGEN, UK)
Buffer RPE	It is a mild washing buffer. Its main function is to remove traces of salts, which are still on the column due to buffers used earlier in the protocol (QIAGEN, UK)

Table 2-5: Components of RNeasy Kits (QIAGEN, UK). The contents and functions of the buffer used in RNeasy Kits.

2.3.5 cDNA synthesis

For cDNA synthesis, the high capacity cDNA reverse transcription kit from Applied Biosystems (UK) was used. Single-stranded cDNA was synthesised from total RNA following the protocol provided by the manufacturer. Preparation of the Master Mix was done on ice. Firstly, 2x Reverse Transcription (RT) Master Mix was prepared and total RNA was added to the 2x RT Master Mix. The components of the final RT master mix were as shown in Table 2-6. The total RNA used was 2 µg per 20 µl of reaction. Reverse transcription was performed in a Thechne TC-3000 PCR thermal cycler (Scientific Laboratory Supplies, UK). Conditions of the thermal cycler set up are shown in Table 2-7.

Component	Volume/Reaction (µL)
10x RT Buffer	2.0
25x dNTP Mix (100mM)	0.8
10x RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNAse inhibitor	1.0
Nuclease-free water	3.2
Total per Reaction	10.0

Table 2-6: Components of the RT master mix using high capacity cDNA reverse transcription kit from Applied Biosystem (UK).

	Step 1	Step 2	Step 3	Step 4
Temperature (ºC)	25	37	85	4
Time	10 minutes	120 minutes	5 minutes	∞

Table 2-7: cDNA synthesis cycle conditions for high capacity cDNA reverse transcription kit (Applied Biosystems).

The resulting cDNA from the RT reaction was collected in RNAse free tubes and stored at -80ºC until further use.

2.3.6 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Fast SYBR® Green Real-Time PCR (Applied Biosystems) was used for all qRT-PCR. The preparation of the reaction master mix was done on ice. TipOne® filters (Starlab) were used to prevent RNAse contamination. Components of the PCR reactions master mixes were as shown in Table 2-8.

Component	Volume for One 20 µl reaction (µl)
Fast SYBR® Green Master Mix (2x)	10.0
Forward and Reverse Primers	Variable*
cDNA template + RNase-free water	Variable**
Total Volume	20.0

Table 2-8: Components of Fast SYBR® Green PCR master mixes; *For forward and reverse primers mix, it was made up to 1µM of final concentration. **For cDNA template, a final diluted concentration of 0.03 µl/µl in RNase-free water was used.

Primers used were designed using the on-line tool Primer-BLAST. Self-complementary and primer specificity to the target RNA were determined. The list of primers is shown in Table 2-9.

RNAs	Primer sequence	Amplicon size (bp)	Design	Annealing temperature (T)
VDR	Forward: 5’CTTCAGGCGAAGCATGAAGC 3’	134	GeneScript (NM_000376.2)	61 and 62.2 ˚C
	Reverse: 5’CCTTCATCATGCCGATGTCC 3’			
CYP2R1	Forward: 5’TGGAGGCATATCAACTGTGGT3’	94	GeneScript (NM_024514.4)	61 and 62.2 ˚C
	Reverse: 5’AAGGCATGGTCTGTCTGCAA3’			
CYP27B1	Forward: 5’AGGCTACCGAGAGTACCACT3’	73	GeneScript (NM_000785.3)	61 and 62.2 ˚C
	Reverse: 5’CAGAAAGCTGGGCGTAGAGG3’			
CYP24A1	Forward: 5’ATGAGCTCCCCCATCAGCAA3’	98	GeneScript (NM_000782.4)	61 and 62.2 ˚C
	Reverse: 5’TACGCCGTAGATGTCACCAG3’			
GAPDH	Forward: 5’TATAAATTGAGCCCGCAGCC3’	143	GeneScript (NM_002046.5)	58.6, 61 and 62.2 ˚C
	Reverse: 5’CGACCAAATCCGTTGACTCC3’			

U6	Forward: 5'CTCGCTTCGGCAGCACA 3'	94	GeneScript (NR_004394.1)	58.6, 61 and 62.2 ˚C
	Reverse: 5'AACGCTTCACGAATTTG C3'			
Collagen I	Forward: 5'GTGCTAAAGGTGCCAAT GGT3'	128	GeneScript (NM_000088.3)	61 and 62.2 ˚C
	Reverse: 5'ACCAGGTTCACCGCTGT TAC3'			
Collagen III	Forward: 5'GTGCTAAAGGTGCCAAT GGT3'	103	GeneScript (NM_000090.3)	59.8, 61 and 62.2 ˚C
	Reverse: 5'ACCAGGTTCACCGCTGT TAC3'			

Table 2-9: qRT-PCR primers and conditions for VDR, CYP2R1, CYP27B1, CYP24A1, collagen I and collagen III amplication and the housekeeping genes U6 and GAPDH.

The final PCR master mix was loaded into 96-well Fast plates (STARLAB) and centrifuged briefly to spin down the contents and eliminate any air bubbles. Finally the PCR reaction plate was run in the Real-Time PCR machine (Applied Biosystems).

2.3.7 Statistical analysis of qRT-PCR results

Analysis Software template iCycleriQ® Real-Time PCR Detection System (Bio-Rad) was used for qPCR data analysis. The analysis was done using the Ct ($Ct^{\Delta\Delta}$) equation method and normalised to the housekeeping gene GAPDH. The resulting analysis data was transferred to Graph Pad Prism (GraphPad, San Diego, CA, USA) or Microsoft Excel (Microsoft Office) for further analysis, construction of graphs and statistical analysis.

2.4 Migration of human epidermal keratinocytes and dermal fibroblasts following mechanical wounding *in vitro* using a scratch wound assay

To determine the effects of $1,25(OH)_2D_3$, cholecalciferol or 25-hydroxyvitamin D_3 on cell migration *in vitro,* a scratch wound assay was used. The age of the donors, passage number and sample code of epidermal keratinocytes and dermal fibroblasts are specified in Table 2-10. Epidermal keratinocytes and dermal fibroblasts were plated into 6-well plates at a cell density of 300,000 cells per well, or 200,000 cells per well respectively. The cells were incubated at 37ºC and 5% CO_2 in air and grown to a confluency of 80%, which takes approximately two days for both keratinocytes and fibroblasts. Culture media was changed every two days for both cell types.

2.4.1 Epidermal keratinocytes and dermal fibroblasts migration

Once epidermal keratinocytes or dermal fibroblasts had reached 80% confluency, they were washed twice with PBS and the media was changed to PKSM (Section 2.1.2.3) or PFSM (Section 2.1.2.5). The keratinocytes/fibroblasts were incubated for a further 24 hours at 37ºC and 5% CO_2 in air. After 24 h, the cells were incubated with 10µg per ml of mitomycin C for 3 hours to inhibit cell proliferation, and determine the true migration rate (O'Toole et al. 1999; Li et al. 2004; Wang et al. 2012; Pomari et al. 2014). A scratch assay was performed using sterile scratch wound device provided by Dr. Kamala which removes a fixed width of 0.8mm of cells (Kamala 2014). To ensure consistency of the scratch, a predesigned template was used as shown in Figure 2-2A. After scratching, cells were washed twice with PBS. 2mLs of fresh PKSM (Section 2.1.2.3)/PFSM (Section 2.1.2.5) with vehicle control (0.01% ethanol) or either 1nM, 10nM or 100nM of $1,25(OH)_2D_3$ / cholecalciferol / 25-hydroxyvitamin D_3 was added to each well. Three replicates were carried out for each of the conditions studied. A CooLpi x4500 Nikon camera was used to take the images at intervals of 0, 4, 8 and 24 hours. A separate template (Figure 2-2B) was used to ensure pictures were taken at fixed points across the scratch wound at each time point. The distance between the wound edges was captured at six fixed points

in triplicate dishes (Figure 2-2B). 24 hours after scratching, total RNA was extracted from the cells to analyse changes in mRNA expression of VDR, CYPs, collagen I and collagen III as described in section 2.3.2 and section 2.3.3.

	Primary epidermal keratinocytes			**Primary dermal fibroblasts**		
Sex	F	F	F	F	F	F
Age	55	59	60	59	59	60
Passage	3	3	3	3	4	3
Sample code	EK2	EK4	EK5	DF7	DF8	DF10

Table 2-10: Primary human epidermal keratinocytes and primary dermal fibroblasts used for scratch assays. F denotes female donor, EK denotes epidermal keratinocyte and DF denotes dermal fibroblast.

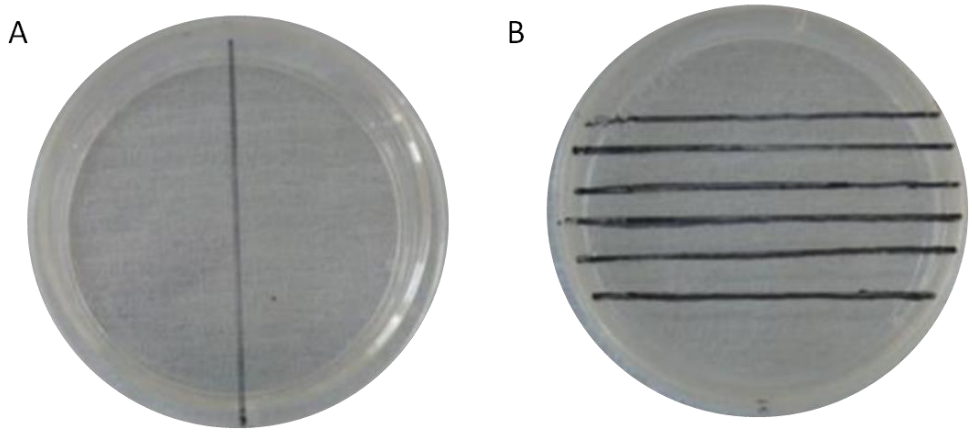


Figure 2-2: Template used in scratch migration assay. (A) A vertical scratch wound was created using a predesigned template. (B) A separate template was created to ensure measurement of distance between wound edges at fixed points in each time point.

2.4.2 Analysis of migration

Images were taken at x125 magnification. The pictures were analysed using Image J. An image of the stage micrometer graticule (Ltd Tonbridge, UK) taken under the same magnification was used for conversion from pixels to mm.

The distance between the wound edges was measured at six fixed points for each well. As each condition was replicated in triplicate dishes, a total of 18 data points were obtained from each condition. The average distance was then calculated at the different time points (0, 8 and 24 hours). The mean migratory distance across the wound edges at different time points was determined. The data was transferred to GraphPad Prism for further statistical analysis.

2.5 Metabolic activity of dermal fibroblasts quantitated by Alamar Blue® assay

An Alamar Blue® assay was performed to assess whether $1,25(OH)_2D_3$ and its precursors modulate dermal fibroblast metabolic activity. Dermal fibroblasts were seeded into 96-well plates (Corning, UK) at a density of 2000 cells/well and allowed to grow to a confluency of 80% in approximately 24 to 48 hours. The age of the donors, passage number and sample code of dermal fibroblasts used are specified in Table 2-11. Dermal fibroblasts were then incubated in PFSM for 24 hours. The PFSM was discarded and the cells washed twice with PBS before the addition of PFSM containing either vehicle control (0.01% absolute ethanol), or 1nM, 10nM or 100nM of either cholecalciferol, 25-hydroxyvitamin D_3 or $1,25(OH)_2D_3$ (total volume of 100 µl). The Alamar Blue® (Thermo Fisher Scientific) reagent (10 µl) was added into each well at 21 hours after incubation with $1,25(OH)_2D_3$ or its precursors. The fluorescence intensity was measured 3 h after adding the Alamar Blue® reagent, using a plate reader at 37ºC (FLUOstar Omega II, BMG Labtech) (excitation: 540nm, emission: 590nm).

Fluorescence intensity values were exported into Microsoft Excel, and the results were analysed by plotting fluorescence intensity against different treatment conditions. Six technical replicates were performed for each of the $1,25(OH)_2D_3$/cholecalciferol/$25(OH)D_3$ treatments at 3 different concentrations. Mean values from four different donors were used for further statistical analysis.

	Primary dermal fibroblasts			
Sex	F	F	F	F
Age	59	59	60	66
Passage	3	3	3	3
Sample code	DF7	DF8	DF10	DF11

Table 2-11: Primary dermal fibroblasts used in the Alamar Blue® assay

2.6 Protein expression by immunocytochemistry

Immunocytochemistry was performed to determine vitamin D receptor (VDR) and alpha-smooth muscle actin (α-SMA) protein expression. The age of the donors, passage number and sample code of dermal fibroblasts used are specified in Table 2-12. Primary fibroblasts were plated in Millicell EZ slide 8-well chamber slides (Figure 2-3) at a cell density of 5,000 cells per well in 500µl PFGM. The media was changed every other day until they reached 70 to 80% confluence, which typically takes 3 to 5 days.

Once the desired confluence was reached, cells were washed twice with PBS the media was changed to PFSM and cells incubated for 24h. A horizontal scratch was made using a 10µL pipette tip and the medium changed to PFSM containing either 10nM of $1,25(OH)_2D_3$, cholecalciferol or 25-hydroxyvitamin D_3. Each of the treatment conditions was done in triplicate (Figure 2-3). The cells were incubated for a further 24 hours before the medium was removed and the cells fixed in 4% formaldehyde (500 µl) for 10 minutes at room temperature. The cells were washed with 0.1% triton in PBS three times (5 minutes each), before 100µl 2% donkey serum in PBS was added to each well for blocking non-specific staining. The cells were incubated at room temperature for an hour before the blocking solution was removed by gently tapping the chamber slides upside down on tissue paper. Then 100µl of primary antibody (Table 2-13) was added to each well and cells were incubated for 4 ˚C overnight in a humidity chamber. For each chamber slide, a well was designated to be the negative control where no primary antibody was used. After incubation

overnight, the primary antibody was removed and the cells washed 3 times with PBS (15 minutes each) before addition of 100µl of secondary antibody (FITC anti-rabbit; Abcam: Ab98502) diluted in PBS (1:200) to each well. The cells were incubated at 37 ˚C for one hour in a dark humidity chamber. The secondary antibody was removed and the cells washed with PBS three times (15 minutes each). Finally the wells were detached from the glass slide following the manufacturer's protocol. Several drops of VECTASHIELD® Mounting Media containing 4',6-diamidino-2-phenylindole (DAPI) (VECTOR laboratories) were added onto the slide before carefully covering with cover slips (22x22 mm; 0.13-0.17mm thick). Finally, the cover slips were fixed to each slide using nail varnish. The dilution factors of the primary antibodies are listed in Table 2-13.

	VDR expression			**α-SMA expression**		
Sex	F	F	F	F	F	F
Age	38	59	60	38	59	60
Passage	3	3	3	4	4	3
Sample code	DF2	DF7	DF10	DF2	DF7	DF10

Table 2-12: Primary human dermal fibroblasts used for VDR and α-SMA expression in immunocytochemistry. F denotes female donor, DF denotes dermal fibroblast.

Protein	Name (type)	Raised in	Manufacturer	Fixation	Blocking	Dilution factor
VDR	VDR antibody	Rabbit	Ab3508 (Abcam)	4% PFA: RT for 10 minutes	2% donkey serum in PBS	1:100
α-SMA	α-SMA antibody	Rabbit	Ab124964 (Abcam)	4% PFA: RT for 10 minutes	2% donkey serum in PBS	1:200

Table 2-13: List of primary antibodies used in immunofluorescence study of VDR and α-SMA.

Figure 2-3: Scratch assay performed in Millicell EZ slide 8-well glasses.

2.6.1.1 Quantification of protein expression

Fluorescence imaging was done using a confocal microscope, the ZEISS AxioVert 200 M with Zeiss AxioCam camera. For FITC, excitation wavelength used was 488 nm and emission wavelength was 514 nm. For DAPI, excitation wavelength used was 364 nm and emission wavelength was 460 nm. Quantitative analysis of protein expression was performed using imageJ software. The values were analysed using Microsoft Excel and final graphs plotted using Prism (GraphPad, San Diego, CA, USA). For each treatment condition, two images per well were taken using the

same laser settings and four single cells in each image were selected randomly for quantitative analysis. The intensity of immunofluorescent staining was calculated using a formula adapted from the work of Gavet *et al* (Gavet and Pines 2010).

Corrected integrated density (corrected for background fluorescence noise) was calculated by subtracting the density of staining signal in the region of interest with the multiplication product of the area of region of interest with mean of background staining signal (Figure 2-4). Mean of background staining signal was the average value of six different measurements of background noise fluorescence (i.e. signal per pixel) around the fibroblast cell of interest (Figure 2-4).

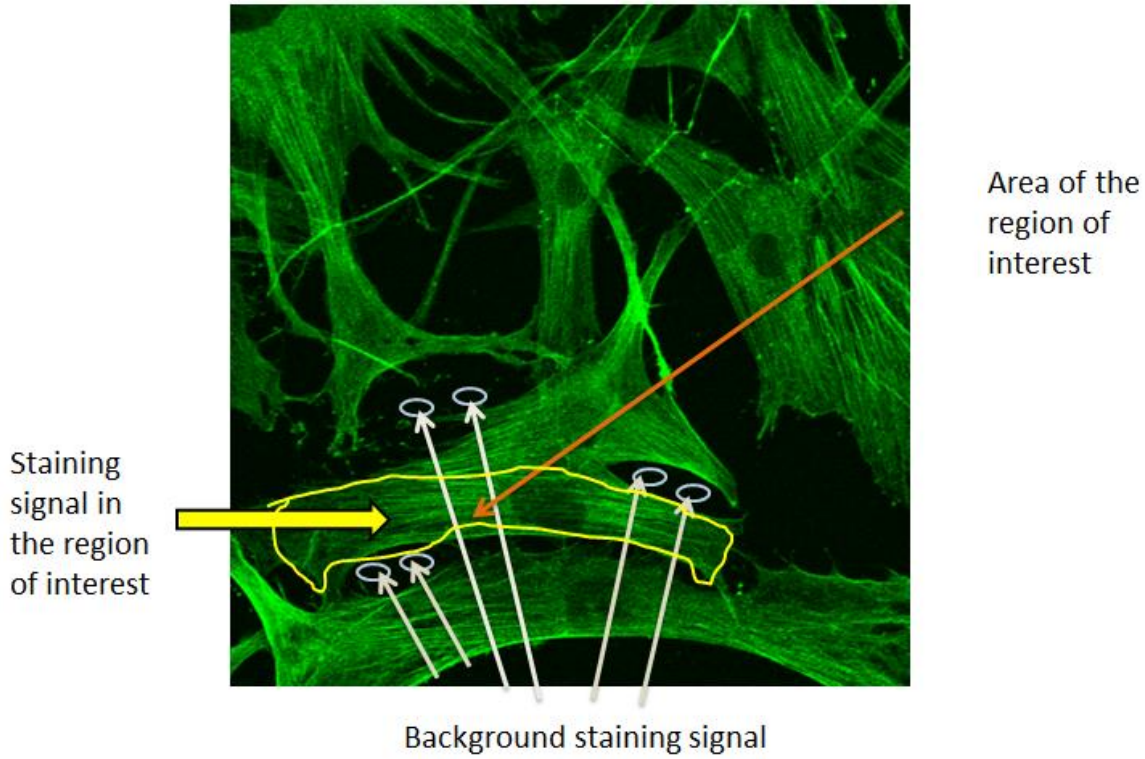


Figure 2-4: Immunofluorescence staining of α-SMA in a human dermal fibroblasts. The yellow arrow is pointing at the staining signal in the region of interest, the orange arrow is showing where the area of region of interest is, and the grey arrow showing the background staining signal of six randomly selected sites around the fibroblast of interest that do not contain any cells.

Corrected integrated density = staining signal density in the region of interest – (Area of the region of interest x mean background staining signal)

2.7 Silencing of VDR using VDR small interfering RNA (siRNA)

Primary dermal fibroblasts were plated into 12-well plates at a density of 20,000 to 30,000 cells per well in 1mL of PFGM. The age of the donors, passage number and sample code of dermal fibroblasts used are specified in Table 2-14. Once they were 80% confluent, the media was changed to PFSM after washing with PBS twice. VDR small interfering RNA (SMARTpool: ON-TARGETplus VDR siRNA from Dharmacon) was used for transfection. Non-target siRNA (Dhamarcon) was used as scramble. siRNA/Scramble-RNA mix and Lipofectamine (Thermo Scientific) mix were prepared separately by diluting them in Opti-MEM® medium (Table 2-15). siRNA/Scramble-RNA mix was then added to Lipofectamine® mix at a 1:1 ratio. The mixture was incubated at room temperature for 5 minutes. Finally, 100µl of the mixture was added to each well that already contained 1ml of PFSM. After 24 hours incubation at 37 ˚C, a scratch assay was performed as described in Section 2.4.1. RNA was then harvested for qRT-PCR to determine the extent of VDR knockdown and changes in the expression of CYP2R1, CYP27B1, CYP24A1 mRNA expression.

	Primary dermal fibroblasts		
Sex	F	F	F
Age	56	60	66
Passage	3	3	3
Sample code	DF7	DF10	DF11

Table 2-14: Primary human dermal fibroblasts used in the silencing experiment for the migration study. F denotes female patient, DF denotes dermal fibroblasts.

siRNA/Scamble RNA mix		**Lipofectamine mix**	
siRNA (15nM)	2.5µl	Lipofectamine® RNAiMAX Reagent	2.5µl
Opti-MEM®	50µl	Opti-MEM®	50µl

Table 2-15: Components of siRNA/Scramble mix and Lipofectamine mix.

2.8 Gelatine zymography to quantify matrix metalloproteinase (MMP) activity

Conditioned media was collected from dermal fibroblasts following the scratch assay as described in Section 2.4.1. The age of the donors, passage number and sample code of dermal fibroblasts used are specified in Table 2-16. Parallel dishes were set up at the same time to collect media from unscratched cells. As a positive control, conditioned medium of fibroblasts treated with Phrobol 12-myristate 13-acetate (PMA) at a final concentration of 100nM (Abcam ab120297) was also included. After 24 hours, the conditioned media was collected and pooled from the triplicate wells before centrifuging (Hettich Rotine 420R) for six minutes at 900g. The supernatant was transferred into a new Falcon tube and stored at -80°C until used in the zymography assays. The conditioned media was thawed out and placed on ice before the start of zymography. The conditioned media was diluted at 1:10 with 2x non-reducing sample buffer (see Appendix 9.1).

Gelatine zymography was used to quantitate the gelatinolytic MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) activity of the conditioned media. The electrophoresis gels were prepared with acrylamide (Sigma-Aldrich, A3449) and containing 1.5 mg/ml gelatin (Sigma-Aldrich, G6650). The separating gel contained 3.75ml of separating gel buffer composed of 1.5M Tris base pH 8.8, 150µl 10% Sodium dodecyl sulphate (SDS), 3.7ml 30% acrylamide, 0.8% bis-acrylamide mix, 75µl 10% ammonium persulfate (APS) (Sigma-Aldrich, UK) and 1.325ml distilled water, while the stacking gel contained 1.875 ml stacking gel buffer 0.5M Tris base pH 6.8, 75µl 10% SDS, 0.975µl 30% acrylamide, 0.8% bis-acrylamide mix, 37.5µl 10% APS (Sigma-Adrich, UK) and 4.58ml distilled water (see Appendix 9.1 for all buffer compositions). Tetramethylethylene diamine (TEMED) (Sigma-Aldrich, T9281) was added to polymerize the gels; 30µl for the separating gel and 15µl for the stacking gel.

The diluted conditioned media was loaded into the gels and the electrophoresis was performed for 110 minutes at 120V in 350ml running buffer. Then the gels were washed three times (each time for 20 minutes in 80ml 12.5x washing buffer for two

gels) with gentle agitation at room temperature. The gels were incubated with 1x incubation buffer to reactivate the gelatinase activity (100ml for each gel), for 24h in an incubator at 37°C (LEEC classic incubator). Gels were then stained with Coomassie brilliant blue staining solution (see Appendix 9.1) (100ml to stain two gels) for 45 minutes at room temperature on an orbital shaker (Denley Orbital Mixer). Images were taken using an UV/white light conversion screen-visualisation system (Bio-Rad, UK). Protease activity was visualised as lytic bands against the blue background. ImageJ software was used to quantitate the percentage change in levels of active MMP-2 and MMP-9 compared to vehicle control.

	Primary dermal fibroblasts		
Sex	F	F	F
Age	59	60	66
Passage	3	3	3
Sample code	DF7	DF10	DF11

Table 2-16: Primary dermal fibroblasts used in gelatine zymography assay.

2.9 *Ex vivo* wound healing assay

An *ex vivo* wound healing assay was used to determine the effects of cholecalciferol and 1,25-dihydroxyvitamin D3 treatment on wound closure in human skin. The assay was adapted from (Stojadinovic and Tomic-Canic 2013; Pellicena 2017). The assay was performed on three individual donors, the first was abdominal skin from a 59-year-old female, the second was breast skin derived from a 34-year-old female and the third was abdominal skin from another 59-year-old female (Table 2-17). Upon receiving the tissue samples in transporting media (Section 2.1.3.1), the skin was washed twice in 1 x PBS in a 60mm tissue culture dish (Sarstedt). The fat tissue was carefully dissected away followed by another wash with PBS. Using a surgical scalpel, the skin sample was cut into smaller pieces of tissue of rectangular shape, each measuring about 1.5cm^2 (1.5cm by length and 1cm by width). The processed skin samples were divided into two groups, providing the non-wounded and wounded groups. For the wounded group, two vertical incisions were made in the epidermis and upper part of the papillary dermis measuring approximately 2mm in width and 2mm in depth. Using forceps and scissors, the epidermis was cut so that a wound was created. The final results produced a 40 mm^3 rectangular wound (Figure 2-5).

In the first *ex vivo* experiment, six groups of wounds from the same donor (n=3 wounds per group) were cultured in either vehicle control (0.01% ethanol), 10% FBS in PFSM (Section 2.1.2.5), 10nM cholecalciferol in PFSM, 100nM cholecalciferol in PFSM containing 10% FBS, 1,25$(OH)_2D_3$ in PFSM, or 1,25$(OH)_2D_3$ in PFSM containing 10% FBS. In the second *ex vivo* experiment, the number of wounds per group was increased (n=8), and skin was cultured in either the vehicle control (0.01% ethanol), 10% FBS in PFSM, 10nM cholecalciferol in PFSM or 10nM 1,25$(OH)_2D_3$ in PFSM. The third *ex vivo* experiment was carried out in the same way as the second *ex vivo* experiment.

The skin explants were cultured at the air-liquid interface to simulate the *in vivo* environment. This was achieved by placing the skin explants in the lid of a 60 mm tissue dish (Sarstedt) (Figure 2-5). In each well, the treatment medium (2 ml) was

changed every day and 200 µl of treatment medium was applied onto the wound area using a pipette at the same time, as the media change.

Images of each skin sample were taken using the digital camera CooLpi x4500 Nikon magnified with the dissecting microscope (Leica) (Figure 2-5) on a daily basis. At either three days or four days after wounding, the *ex* vivo wounds from each sub-group were frozen down rapidly by immersing in liquid nitrogen followed by storage in the -80ºC freezer. At day 7, all remaining skin samples were frozen down in the same way. Frozen samples were processed by cryosectioning (10µm) and haematoxylin and eosin (H&E) staining was used for histology.

For H&E staining, the slides were thawed and air dried for several minutes to remove moisture. They were then fixed with acetone at -20ºC for 3 minutes. The slides were washed with PBS for 10 minutes before staining with 0.1% Mayer's Haematoxylin (Sigma-Aldrich, UK) for 10 minutes in a 50 ml conical tube. The slides were transferred to a Coplin jar and were rinsed in cool running distilled water for 5 minutes. They were then dipped in Eosin Y solution (Sigma-Aldrich, UK) for 30 seconds. This is followed by dipping the slides in distilled water for 2 minutes. The slides were then equilibrate in 70% ethanol and 90% ethanol for 30 seconds each, followed by 100% ethanol for 1 minute. Subsequently, the slides were dipped in 3 changes of xylene, up to 5 minutes each. Lastly, the stained tissue sections were mounted with HistoMount (Thermo Fisher Scientific).

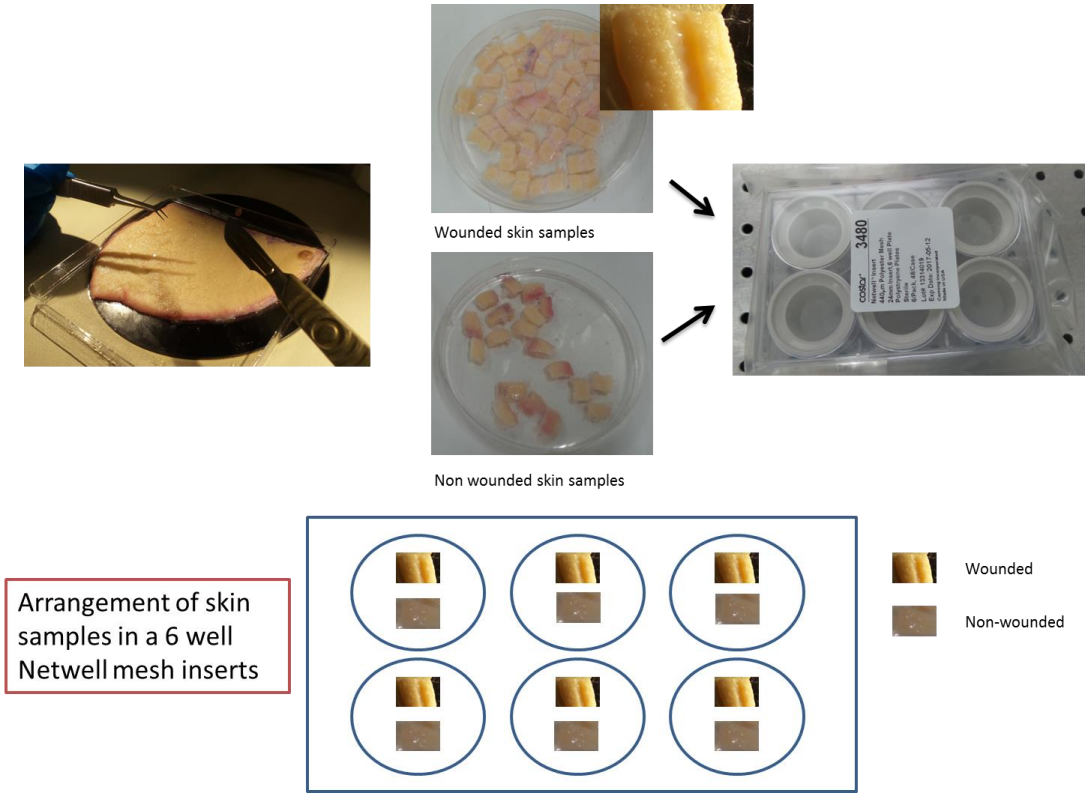


Figure 2-5: Ex vivo wound healing model using skin from abdominoplasty and breast reduction surgery.

The wound area of each skin sample at each time point (0, 3, 4 and 6 days) was analysed using ImageJ. Rate of wound closure was calculated. The results were transferred to GraphPad Prism for further statistical analysis.

Patient number	Gender	Age (years)	Anatomical source of skin explants	Number of wounds per treatment group at day 3 or 4	Number of wounds per treatment group at day 6
1	Female	59	Abdomen	3	2
2	Female	34	Breast	8	5
3	Female	59	Abdomen	8	5

Table 2-17: Demographic data and sources of skin explants and number of wounds per treatment condition.

2.10 Statistical Analysis

Statistical analysis of the results obtained from the experiments were performed to examine the quantitative data, interpret the differences in the observed data and test the statistical significance of the findings, in order to accept or reject a null hypothesis (Nayak and Hazra 2011). In this work, statistical values were obtained using Microsoft Office Excel 2010 and GraphPad Prism Version 6.01.

In experiments involving two groups, normal Gaussian distribution of the data from two groups was confirmed using the Kolmogorov-Smirnov test. When normally distributed, the difference between each donor mean was analysed using either paired or unpaired (parametric) Student's t-test to compare means. If the data was not normally distributed, the Mann Whitney U-test (non-parametric) was used instead to compare medians (Hart 2001).

When more than two groups of samples were involved, the analysis of variance (ANOVA) test was used. This allows comparison of any number of sample means in one single test. One-way ANOVA was used to analyse the effect of one variable over the sample mean of different treatment groups while two-way ANOVA was used to analyse the effects of two independent variables on a continuous dependent variable (Nayak and Hazra 2011). The Bonferroni test was used as the post-hoc test.

Significance testing of the results was assessed using the p-value (Dahiru 2008). A p-value of less than 0.05 was considered statistically significant. A p value of equal or below 0.05 (denoted by * in the result section), 0.01 (denoted by **) and 0.001 (denoted by ***) were used in this study.

3 RESULTS

3.1 A comparison of the relative mRNA expression of VDR, CYP2R1, CYP27B1, and CYP24A1 in cultured human epidermal keratinocytes (EKs) and dermal fibroblasts (DFs)

3.1.1 Dermal fibroblasts expressed higher levels of VDR mRNA than epidermal keratinocytes; while epidermal keratinocytes expressed higher level of vitamin D_3 metabolising enzymes, in both donor-matched and non donor-matched samples derived from facial skin of female donors.

In donor-matched samples (n=3), primary cultures of epidermal keratinocytes and dermal fibroblasts were derived from female donors with a mean age of 58 (Table 2-2). In non donor-matched samples, the mean age was 57 years (Table 2-2). All cells were analysed at passage 2-5.

It was confirmed that primary cultures of epidermal keratinocytes and human dermal fibroblasts both expressed VDR and all the CYPs for vitamin D_3 metabolism. Cultured human dermal fibroblasts expressed a higher level of VDR mRNA compared to epidermal keratinocytes (Figure 3-1 and Figure 3-2). In donor-matched samples, it was 22-fold higher (Figure 3-1). On the other hand, epidermal keratinocytes expressed higher levels of CYP2R1, CYP27B1 and CYP24A1 (Figure 3-1 and Figure 3-2). There was variability in GAPDH Ct values between donor samples, up to a maximum of 1 cycle.

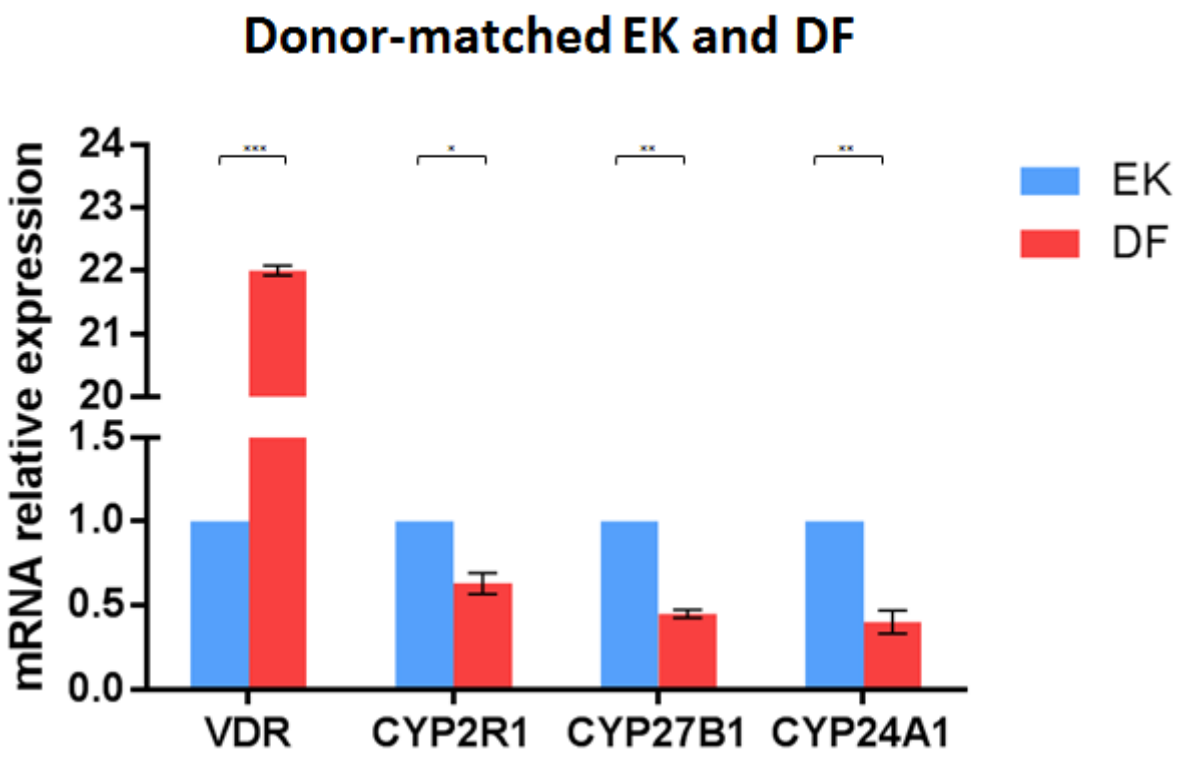


Figure 3-1: The expression of VDR, CYP2R1, CYP27B1 and CYP24A1 in donor-matched primary cultures of human epidermal keratinocytes and dermal fibroblasts. Relative expression of mRNA in donor-matched EKs and DFs (n=3 donors) ± SEM quantitated by RT-qPCR. Relative expression level in DFs was normalised against donor-matched EKs. *Denotes $p<0.05$, ** denotes $p<0.01$ and *** denotes $p<0.001$ in two-way ANOVA.

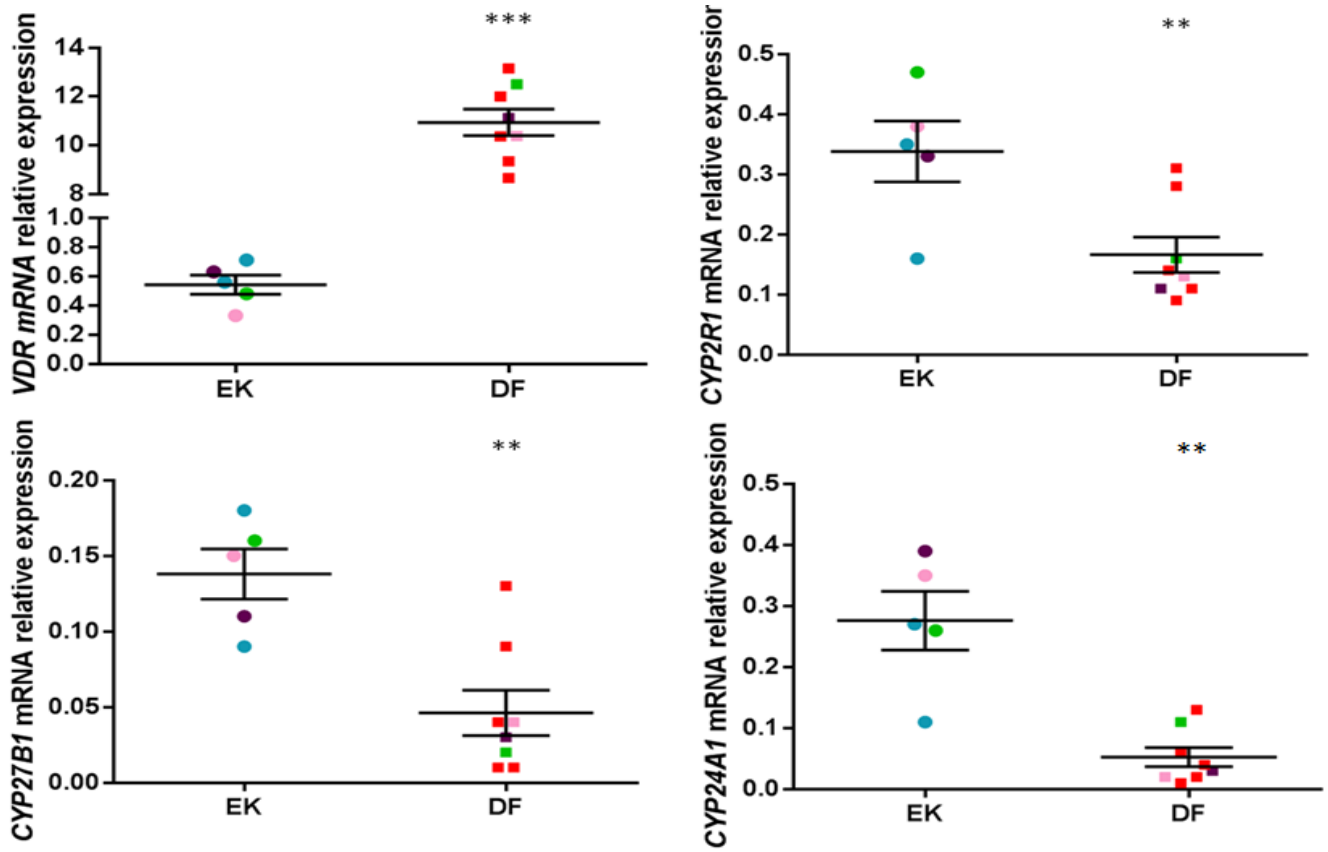


Figure 3-2: Relative expression of VDR and CYPs mRNA in the unmatched EKs (n=5, indicated by a circle) and DFs (n=8, indicated by squares) ± SEM quantitated by RT-qPCR. (A) VDR mRNA expression, (B) CYP2R1 mRNA expression, (C) CYP27B1 mRNA expression and (D) CYP24A1 mRNA expression. The relative expression was normalised against the housekeeping gene, GAPDH. Matched samples were: EK2 and DF4 (purple), EK4 and DF7 (green); EK5 and DF10 (pink). The rest of the samples were unmatched (blue and red). **Denotes $p<0.01$ and ***denoted $p<0.001$ in unpaired Student's t-test.

3.2 $1,25(OH)_2D_3$ modulates migration of human epidermal keratinocytes and dermal fibroblasts in culture

3.2.1 $1,25(OH)_2D_3$ stimulates migration of human epidermal keratinocytes in a scratch wound assay.

To assess the effect of $1,25(OH)_2D_3$ on cell migration, a scratch wound assay was carried out. All epidermal keratinocytes were derived from facial skin of female donors and assayed at passage number 3 (Table 2-10). The mean age of donors was 58 years. Scratch wound assays were conducted for up to 24 hours. Mitomycin C was used to block keratinocyte proliferation so that only migration was observed.

The migratory distance of keratinocytes from the edges of the scratch wound was measured and analysed at 4h, 8h and 24h after the initial scratch, between fixed points using the template described in Section 2.4.1 (Figure 2-2B). Images of epidermal keratinocytes at 0 and 24 hours are shown in Figure 3-3A. At 4 and 8 hours, $1,25(OH)_2D_3$ had no effect on epidermal keratinocyte migration. However, by 24 hours, $1,25(OH)_2D_3$ had accelerated epidermal keratinocyte migration at all three concentrations (Figure 3-3B).

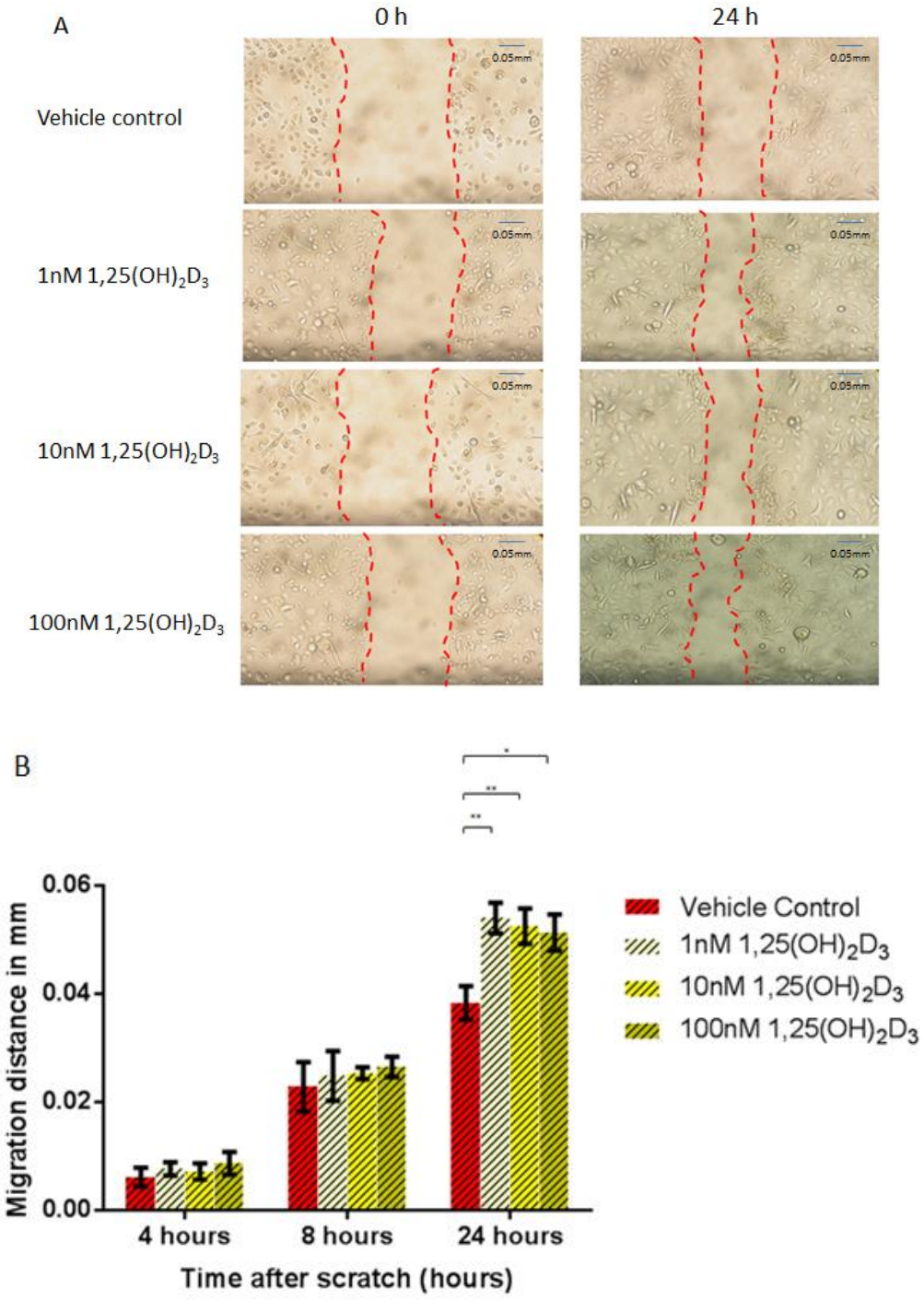


Figure 3-3: $1,25(OH)_2D_3$ accelerates epidermal keratinocytes migration in a scratch wound assay. (A) Representative images of epidermal keratinocytes incubated with vehicle control (0.01% ethanol) or 1nM/10nM/100nM of $1,25(OH)_2D_3$. (B) Analysis of migration was performed after 4, 8 and 24 hours. Cells were assayed in triplicate wells, with six measurements per well. Data presented as mean (n=3 donors) ± SEM *Denotes $p<0.05$, **denotes $p<0.01$ using two-way ANOVA.

3.2.2 1,25(OH)$_2$D$_3$ inhibits migration of human dermal fibroblasts in a scratch wound assay.

All dermal fibroblasts were derived from facial skin of female donors and assayed between passage number 3-4 (Table 2-10). The mean age of donors was 59 years. Scratch wound assays were conducted for up to 24 hours. Mitomycin C was used to inhibit fibroblasts proliferation so that true migration could be observed.

The migratory distance of fibroblasts from the edges of the scratch wound was measured and analysed at 4h, 8h and 24h following scratching, between fixed points using the template described in Section 2.4.1 (Figure 2-2B). In the presence of 10% FBS, incubation with 1,25(OH)$_2$D$_3$ had no effect on dermal fibroblast migration up to 24 hours. At 24 hours, migration was inhibited by 1,25(OH)$_2$D$_3$ at all three concentrations (Figure 3-4B). Images of dermal fibroblasts at 0 and 24 hours are shown in Figure 3-4A.

To assess whether the presence of serum masks the effect of migration or possibly reduces bio-availability of 1,25(OH)$_2$D$_3$, migration assays were repeated in serum free media. In serum free media, incubation with 1,25(OH)$_2$D$_3$ at all three concentrations inhibited dermal fibroblast migration as early as 4 hours and for up to 24 hours (Figure 3-5B). Images of dermal fibroblasts at 0 and 24 hours are shown in Figure 3-5A.

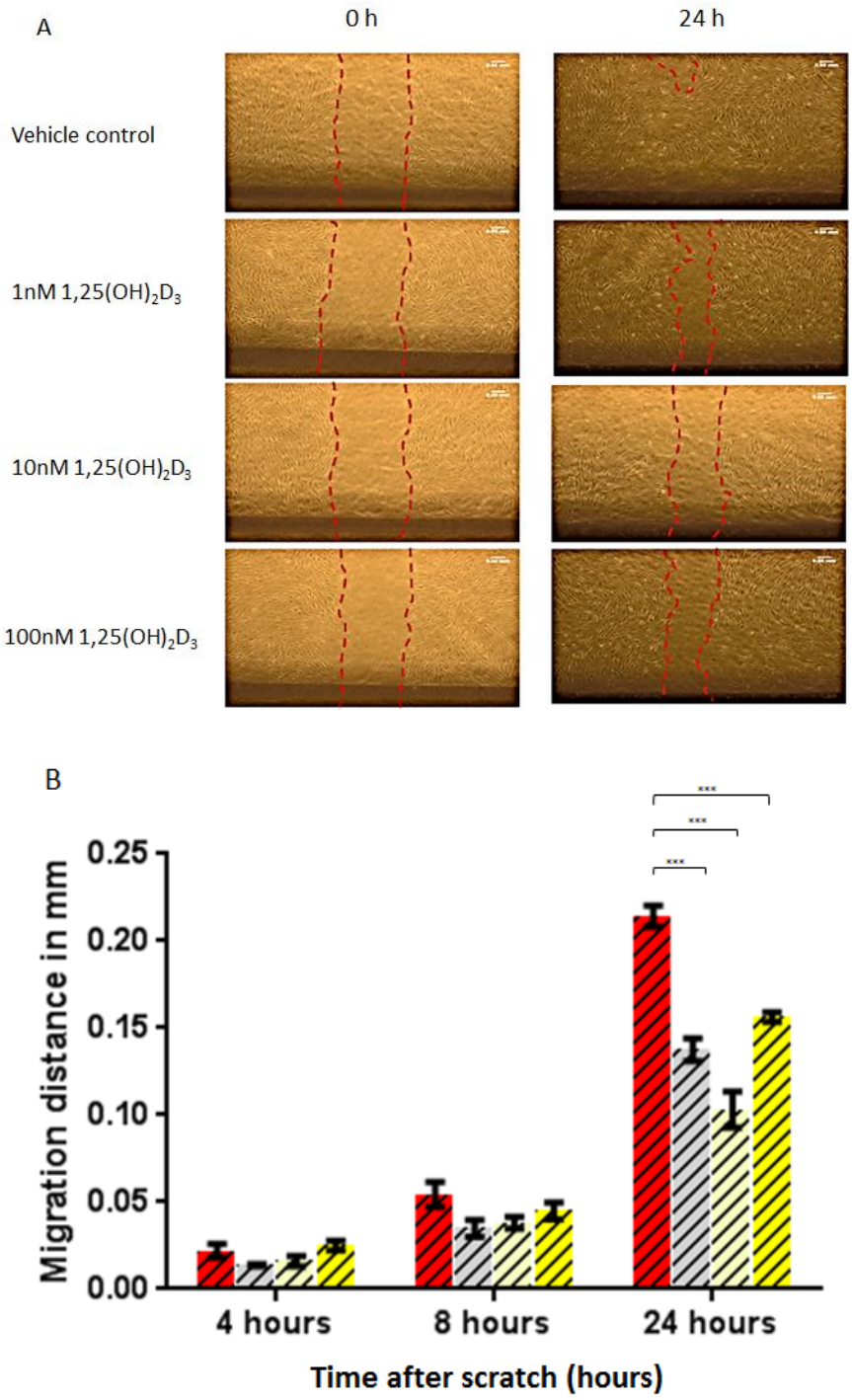


Figure 3-4: In the presence of serum, $1,25(OH)_2D_3$ inhibits dermal fibroblast migration in a scratch wound assay after 24 hours. (A) Representative images of dermal fibroblasts incubated with vehicle control (red hatched bar-0.01% ethanol) or 1nM/10nM/100nM (grey/light yellow/dark yellow hatched bars) of $1,25(OH)_2D_3$. (B) Analysis of migration was performed after 4, 8 and 24 hours. Cells were assayed in triplicate wells, with six measurements per well. Data presented as mean (n=3 donors) ± SEM. ***Denotes $p<0.001$ using two-way ANOVA.

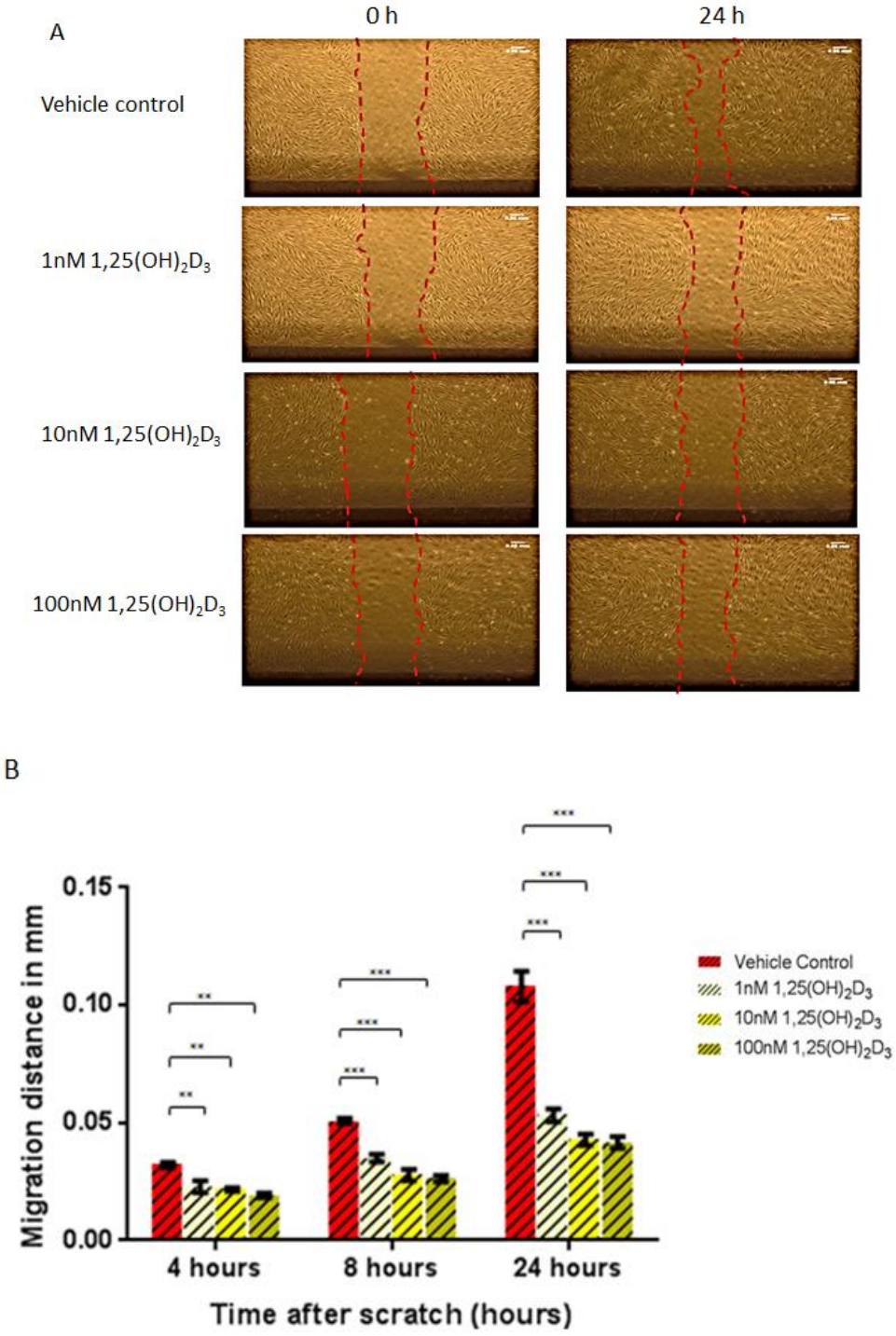


Figure 3-5: $1,25(OH)_2D_3$ inhibits dermal fibroblast migration in serum free media as early as 4 hours and for up to 24 hours. (A) Representative images of dermal fibroblasts incubated with vehicle control (0.01% ethanol) or 1nM/10nM/100nM of $1,25(OH)_2D_3$. (B) Analysis of migration was performed after 4, 8 and 24 hours. Cells were assayed in triplicate wells, with six measurements per well. Data presented as mean (n=3 donors) ± SEM. **Denotes $p<0.01$ and ***denotes $p<0.001$ using two-way ANOVA.

3.2.3 Precursors of 1,25$(OH)_2D_3$ did not modulate migration of cultured human dermal fibroblasts in a scratch wound assay.

Incubation with cholecalciferol (1-100nM) did not modulate migration of dermal fibroblasts at any time points measured up to 24 hours in either media containing 10% FBS or serum free media (Figure 3-6A and Figure 3-6B).

Similarly, incubation with 25$(OH)D_3$ (1-100nM) did not modulate migration of dermal fibroblasts at any of the time points up to 24 hours in either media containing 10% FBS or serum free media (Figure 3-6C and Figure 3-6D).

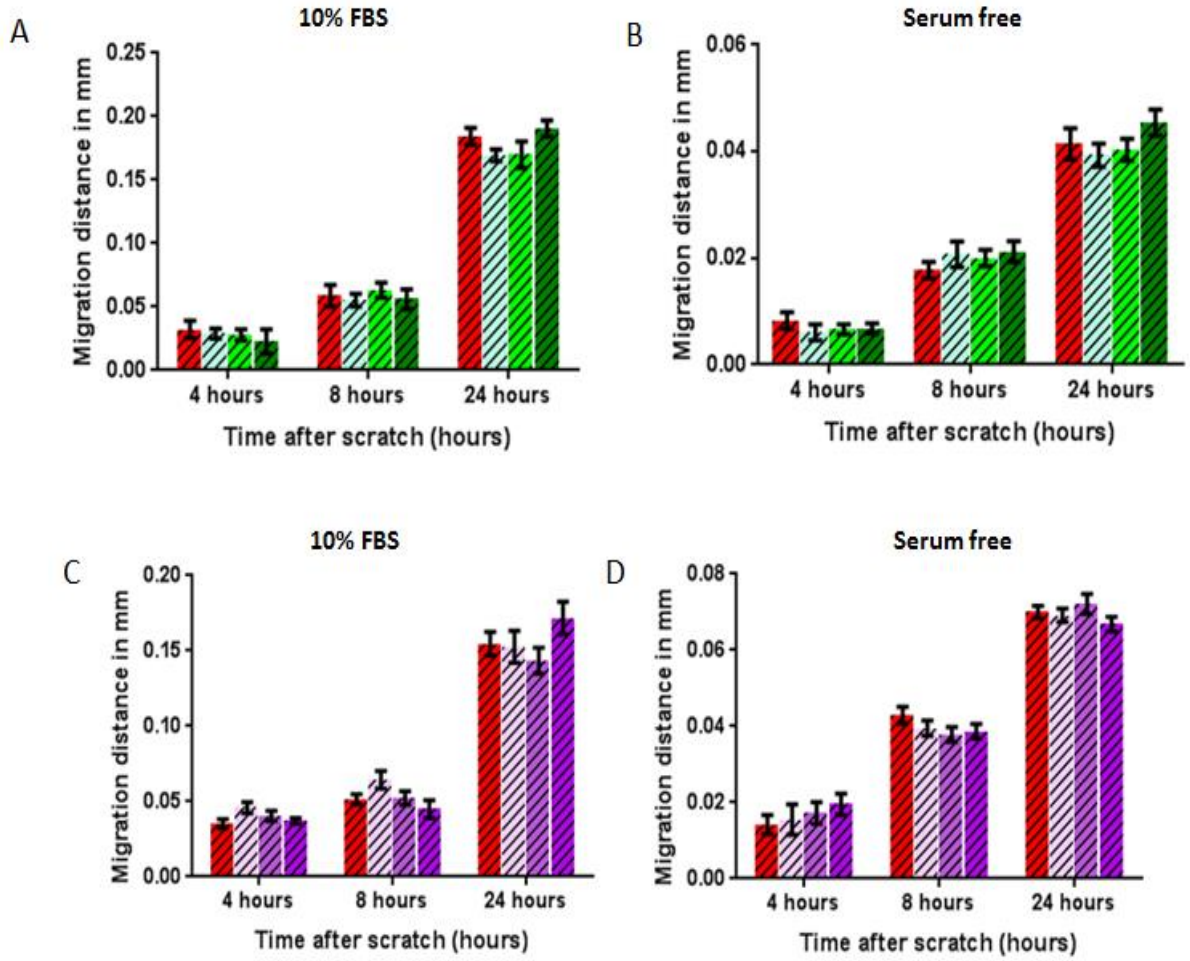


Figure 3-6: Cholecalciferol and 25(OH)D_3 do not modulate cultured dermal fibroblasts migration in a scratch wound assay. Migration distance of dermal fibroblasts at 4, 8 and 24 h after scratching, incubated with (A) vehicle control (0.01% ethanol) in 10% FBS or 1/10/100nM cholecalciferol in 10% FBS (B) vehicle control (0.01% ethanol) in serum free media or 1/10/100nM cholecalciferol in serum free media (C) vehicle control (0.01% ethanol) in 10% FBS or 1/10/100 25(OH)D_3 in 10% FBS (D) vehicle control (0.01% ethanol) in serum free media or 1/10/100 25(OH)D_3 in serum free media. Vehicle control (red), 1/10/100nM cholecalciferol (light green to dark green), 1/10/100nM 25(OH)D_3 (light purple to dark purple). Data presented as mean (n=3 donors) ± SEM.

3.3 Incubation with $1,25(OH)_2D_3$ or its precursors did not modulate metabolic activity of human dermal fibroblasts after 24 hours

An Alamar Blue Assay was used to assess the effect of $1,25(OH)_2D_3$ and its precursors on metabolic activity of human dermal fibroblasts in a scratch wound assay. Dermal fibroblasts were derived from facial skin of four female donors, with a mean age of 61 years (Table 2-11).

$1,25(OH)_2D_3$, cholecalciferol and $25(OH)D_3$ did not modulate metabolic activity of dermal fibroblasts after 24 hours incubation, under either non-scratched or scratched conditions (Figure 3-7).

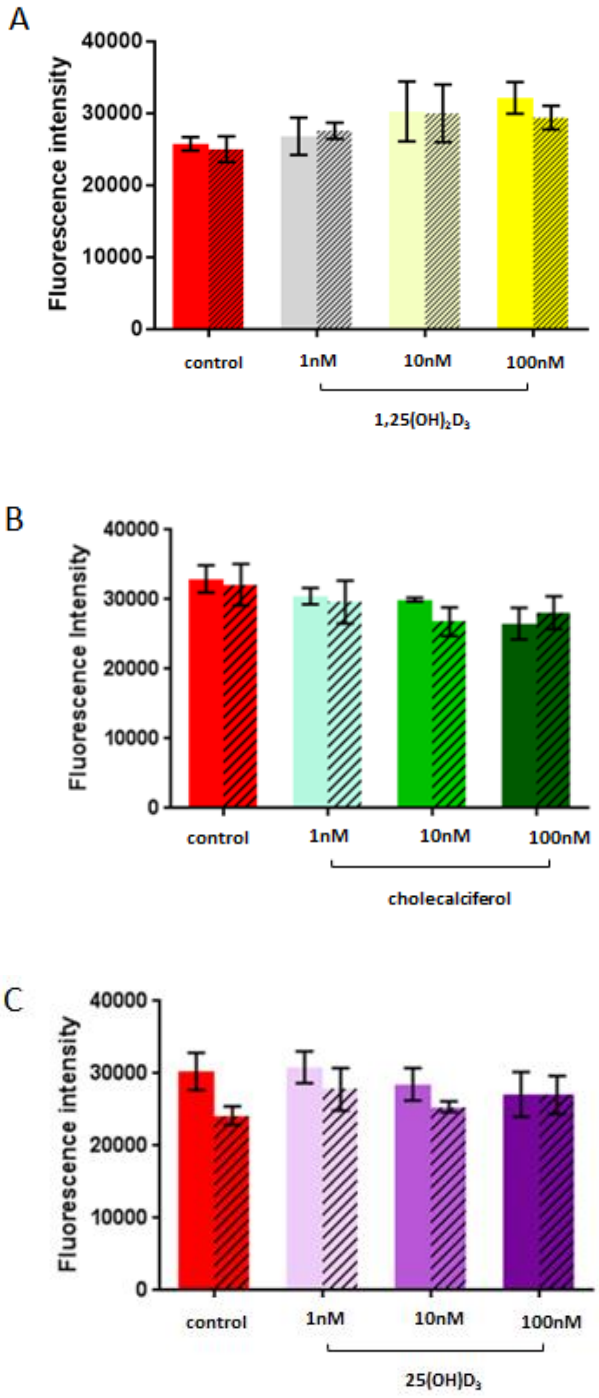


Figure 3-7: Fluorescence intensity reading from the Alamar Blue Assay, representative of the relative metabolic activity of dermal fibroblast, after 24 h incubation of either non-scratched or scratched dermal fibroblasts, in (A) vehicle control (0.01% ethanol), 1/10/100nM 1,25(OH)$_2$D$_3$; (B) vehicle control (0.01% ethanol), 1/10/100nM cholecalciferol; (C) vehicle control (0.01% ethanol), 1/10/100nM 25-hydroxyvitamin D$_3$. Solid bar = non-scratched dermal fibroblasts, hatched bar = scratched dermal fibroblasts. Data was presented as the mean of four donors ± SEM.

3.4 Nuclear VDR protein expression was upregulated only in scratched dermal fibroblasts, in the presence of $1,25(OH)_2D_3$

All dermal fibroblasts were derived from facial skin of female donors and assayed at passage 3 (Table 2-12). The median age of donors (n=3) was 59 years. At basal conditions, VDR was found localised mainly in the perinuclear region of dermal fibroblasts (Figure 3-8A, Figure 3-9A, and Figure 3-10A).

While incubation with $1,25(OH)_2D_3$ did not alter the nuclear protein expression of VDR in unscratched dermal fibroblasts, in mechanically wounded dermal fibroblasts incubation with 10nM of $1,25(OH)_2D_3$ significantly upregulated the nuclear protein expression of VDR (Figure 3-8). However, nuclear protein expression of VDR in unscratched and scratched dermal fibroblasts were unchanged after 24 hours incubation with either 10nM cholecalciferol or 10nM $25(OH)D_3$ (Figure 3-9 and Figure 3-10).

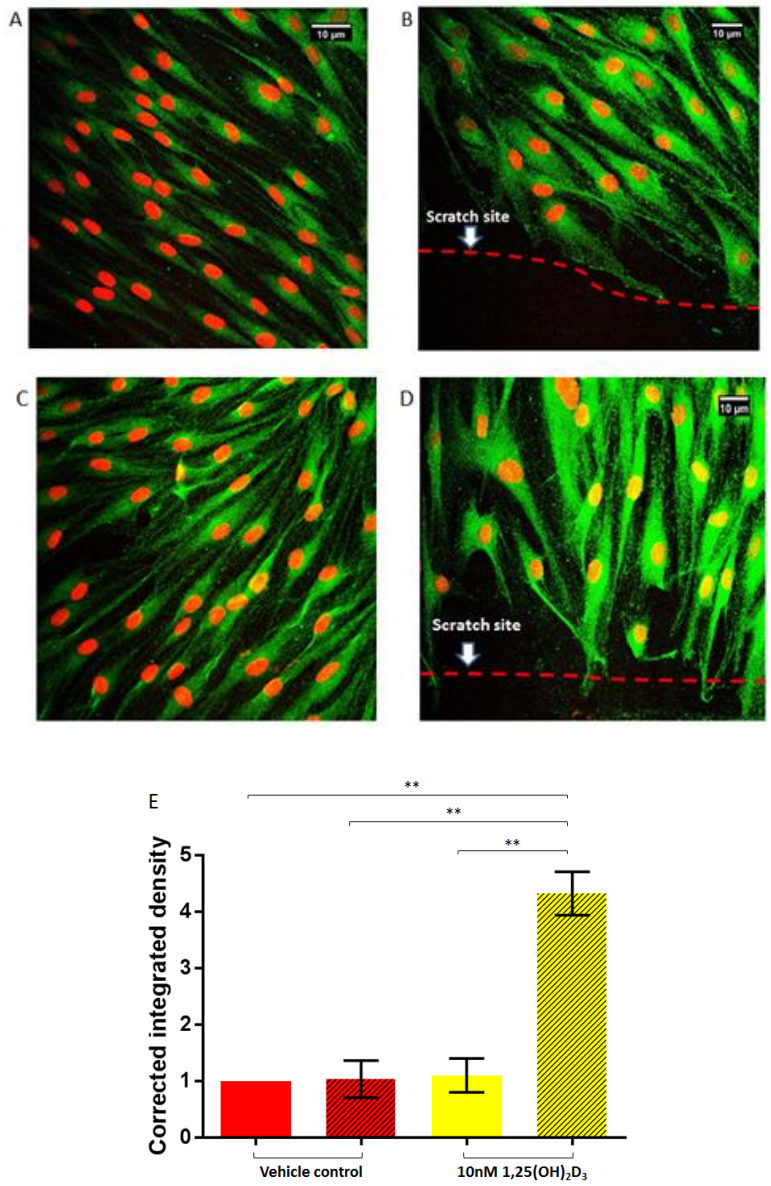


Figure 3-8: $1,25(OH)_2D_3$ upregulates nuclear VDR expression in scratched human dermal fibroblasts. Vitamin D receptor (VDR) immunofluorescent staining (green) with nuclear DAPI staining (red) in non-scratched and scratched dermal fibroblasts, incubated with or without 10nM $1,25(OH)_2D_3$ for 24 hours. (A) vehicle control (0.01% ethanol): non-scratched dermal fibroblasts; (B) vehicle control (0.01% ethanol): scratched dermal fibroblasts; (C) 10nM $1,25(OH)_2D_3$: non-scratched dermal fibroblasts; (D) 10nM $1,25(OH)_2D_3$: scratched dermal fibroblasts. (E) Corrected integrated density of nuclear staining of VDR in non-scratched (non-hatched bars) and scratched (hatched bars) dermal fibroblasts, in the absence (red) or the presence (yellow) of 10nM $1,25(OH)_2D_3$. Data are presented as patient mean (n=3 donors), normalised against (A) ± SEM. **Denotes $p<0.01$ using one-way ANOVA test. Magnification = 20x. Scale bar = 10 µm

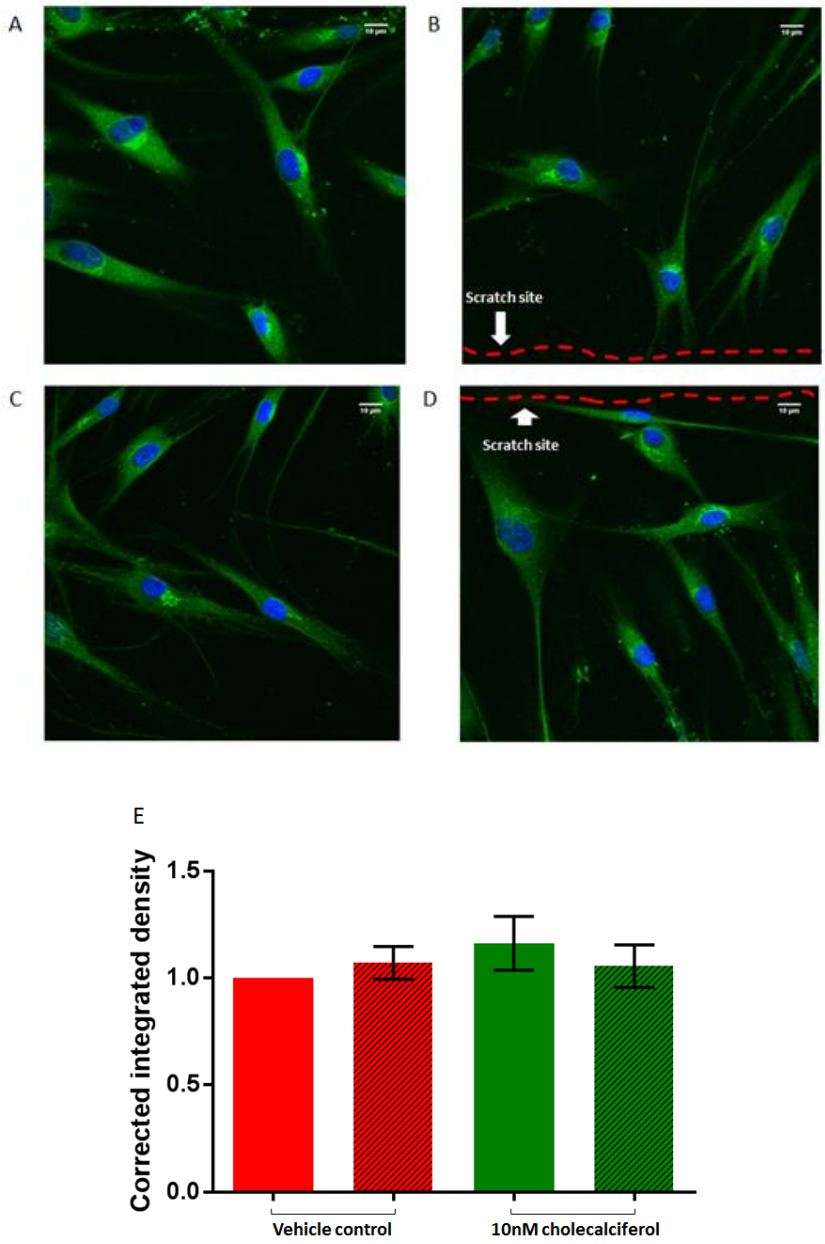


Figure 3-9: Cholecalciferol does not modulate nuclear VDR expression. Vitamin D receptor (VDR) immunofluorescent staining (green) with nuclear DAPI staining (blue) in non-scratched and scratched dermal fibroblasts, incubated with or without 10nM cholecalciferol for 24 hours. (A) vehicle control (0.01% ethanol): non-scratched dermal fibroblasts; (B) vehicle control (0.01% ethanol): scratched dermal fibroblasts; (C) 10nM cholecalciferol: non-scratched dermal fibroblasts; (D) 10nM cholecalciferol: scratched dermal fibroblasts. (E) Corrected integrated density of nuclear staining of VDR in non-scratched (non-hatched bars) and scratched (hatched bars) dermal fibroblasts, in the absence (red) or the presence (green) of 10nM choleclaciferol. Data are presented as patient mean (n=3 donors), normalised against (A) ± SEM. Magnification = 40x. Scale bar = 10 µm

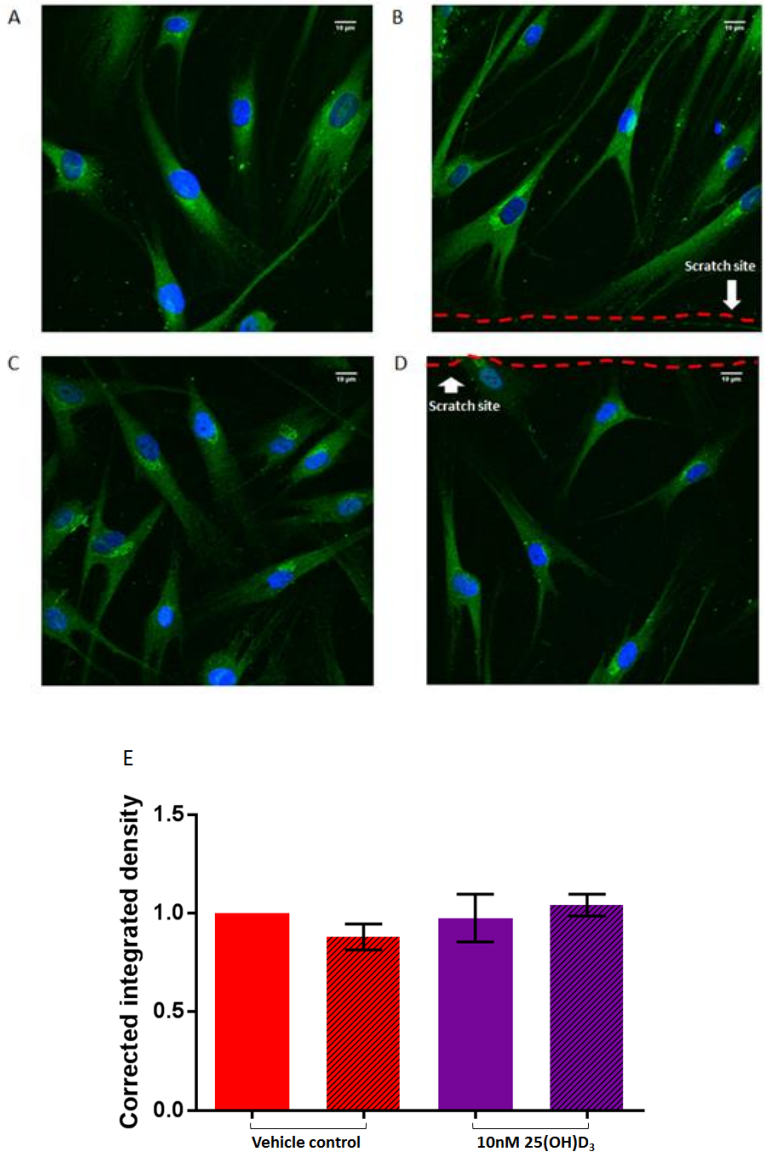


Figure 3-10: 25(OH)D_3 does not modulate nuclear VDR expression. Vitamin D receptor (VDR) immunofluorescent staining (green) with nuclear DAPI staining (blue) in non-scratched and scratched dermal fibroblasts, incubated with or without 10nM 25(OH)D_3 for 24 hours. (A) vehicle control (0.01% ethanol): non-scratched dermal fibroblasts; (B) vehicle control (0.01% ethanol): scratched dermal fibroblasts; (C) 10nM 25(OH)D_3: non-scratched dermal fibroblasts; (D) 10nM 25(OH)D_3: scratched dermal fibroblasts. (E) Corrected integrated density of nuclear staining of VDR in non-scratched (non-hatched bars) and scratched (hatched bars) dermal fibroblasts, in the absence (red) or the presence (purple) of 10nM 25(OH)D_3. Data are presented as patient mean (n=3 donors), normalised against (A) ± SEM. Magnification = 40x. Scale bar = 10 µm

3.5 Knockdown of VDR abolished the inhibitory effect of 1,25(OH)$_2$D$_3$ on human dermal fibroblast migration

All dermal fibroblasts were derived from facial skin of female donors and assayed at passage 3 (Table 2-13). The median age of donors was 61 years. VDR siRNA transfection was successful with efficiency between 89-91% (Figure 3-11). Silencing of VDR resulted in abolishment of the inhibitory effects of 10nM 1,25(OH)$_2$D$_3$ on dermal fibroblasts migration up from 4 hours and for up to 24 hours (Figure 3-12A and Figure 3-12B).

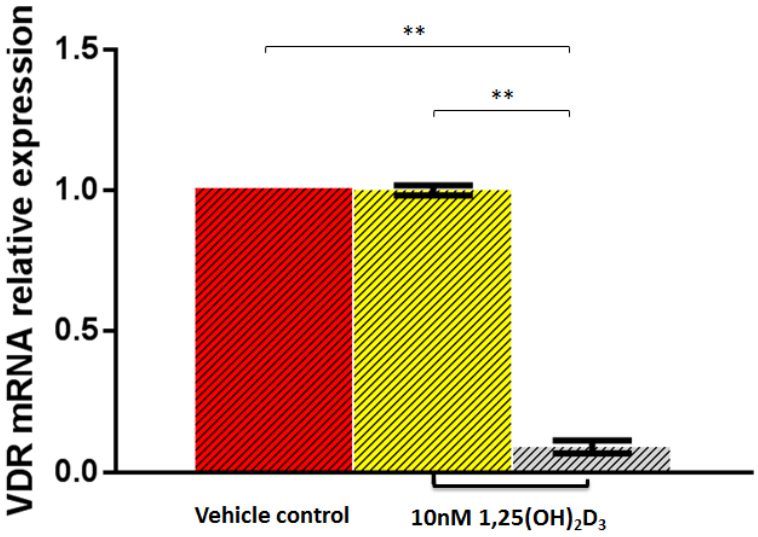


Figure 3-11: VDR siRNA transfection achieved VDR knockdown efficiency of 89-91% in scratched dermal fibroblasts. Quantitative comparison of mRNA expression levels for VDR in vehicle control (red hatched); 10nM 1,25(OH)$_2$D$_3$ and non-target siRNA control (yellow hatched); 1,25(OH)$_2$D$_3$ and VDR siRNA (grey hatched). All assays were performed in triplicate. Data are presented as patient mean (n=3 donors) ± SEM. **Denotes $p<0.01$ using one-way ANOVA test.

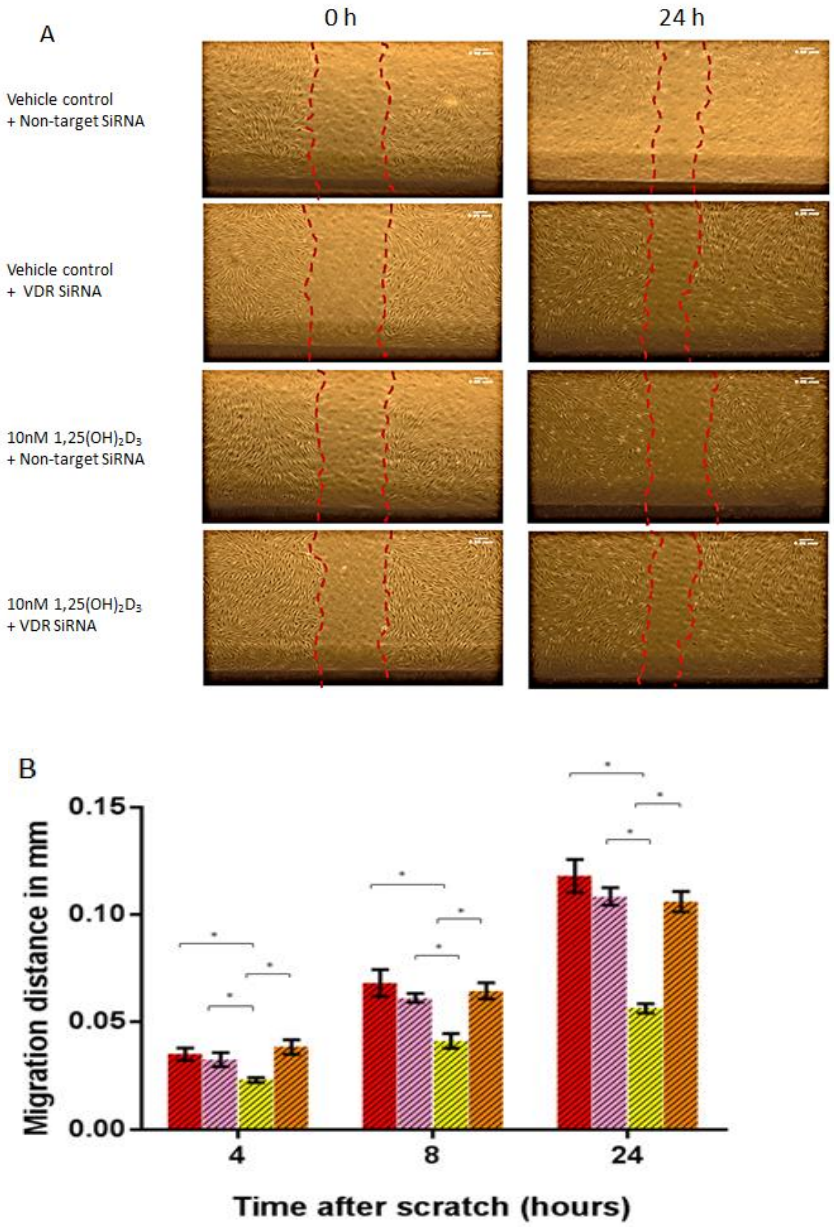


Figure 3-12: VDR knockdown with siRNA abolished the inhibitory effect of $1,25(OH)_2D_3$ on dermal fibroblast migration in a scratch wound assay. (A) Representative images of dermal fibroblasts incubated with, or without 10nM $1,25(OH)_2D_3$, transfected with either non-target siRNA or VDR siRNA. Red: vehicle control + non-target siRNA; pink: vehicle control + VDR siRNA; yellow: with $1,25(OH)_2D_3$ + non-target siRNA; orange: with $1,25(OH)_2D_3$ + VDR siRNA (B) Analysis of migration was done at 4, 8 and 24 hours after scratching. Cells assayed in triplicate wells, with six measurements per well. Data presented as mean (n=3 donors) ± SEM. *Denotes $p<0.05$ using two-way ANOVA.

3.6 Mechanical wounding did not modulate relative mRNA expression of VDR, CYP2R1, CYP27B1 and CYP24A1 mRNA in epidermal keratinocytes and dermal fibroblasts.

In order to understand why there are differences in the migratory response of epidermal keratinocytes and dermal fibroblasts to $1,25(OH)_2D_3$, mechanical wounding (scratching) of monolayers of epidermal keratinocytes and dermal fibroblasts was performed to investigate whether it affects mRNA expression of VDR, CYP2R1, CYP27B1 and CYP24A1. Both the keratinocytes and fibroblasts were derived from facial skin of female donors and assayed between passages 3 to 5. The mean age of donors for keratinocytes (n=5) and fibroblasts (n=8) was 57 years (Table 2-2).

There was no significant change in the transcriptional activities of vitamin D receptor (VDR) or the vitamin D_3 metabolising enzymes 24 hours after mechanical wounding in either cell type in the absence of any of the vitamin D_3 ligands (Figure 3-13). To determine whether the presence of $1,25(OH)_2D_3$ modulated the expression of the VDR or the CYP enzymes, further studies were performed in the presence of $1,25(OH)_2D_3$, cholecalciferol and $25(OH)D_3$.

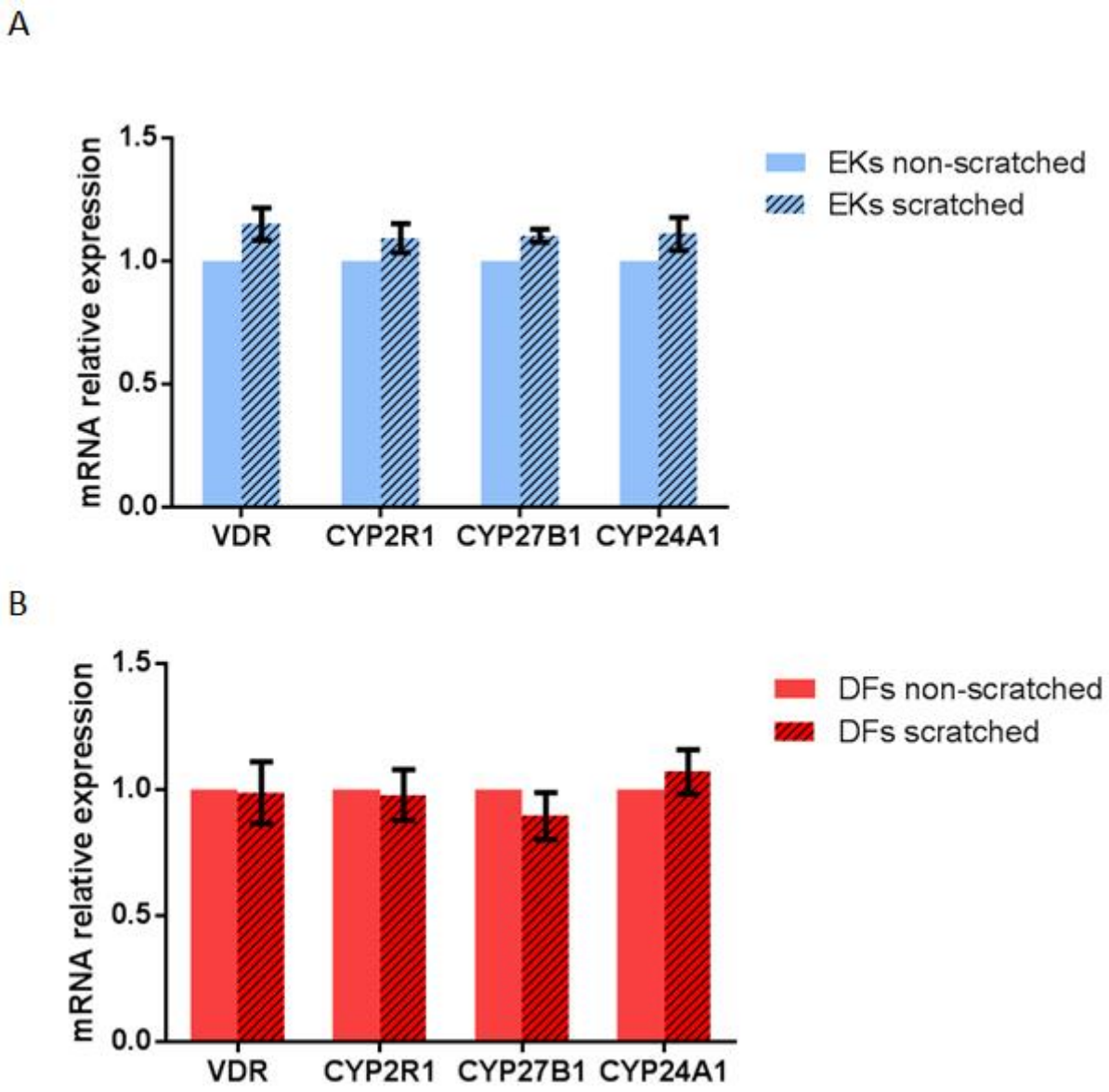


Figure 3-13: Mechanical wounding of cultured epidermal keratinocytes and dermal fibroblasts does not modulate VDR and CYP transcriptional activities in a scratch wound assay. Relative mRNA expression of VDR, CYP2R1, CYP27B1, and CYP24A1 in non-scratched and scratched (A) epidermal keratinocytes (n=5 donors) (B) Dermal fibroblasts (n=8 donors), quantified by qRT-PCR. Relative expression for scratched epidermal keratinocytes/dermal fibroblasts were normalised against non-scratched epidermal keratinocytes/dermal fibroblasts. For each donor, the assay was performed in triplicates. Data was presented as the mean relative expression normalised against GAPDH ± SEM.

3.7 $1,25(OH)_2D_3$ and $25(OH)D_3$ increased CYP24A1 mRNA expression in epidermal keratinocytes and dermal fibroblasts, but did not modulate VDR mRNA expression. Cholecalciferol increased VDR and all the CYP enzymes mRNA expression in scratched dermal fibroblasts. In non-scratched dermal fibroblasts, only CYP27B1 expression was upregulated by cholecalciferol.

All epidermal keratinocytes and dermal fibroblasts were derived from facial skin of female donors with a mean age of 58 years and 59 years respectively. All cells were assayed at passage 3-4 (Table 2-10). There was variability between GAPDH Ct values between donors, up to a maximum of 1 cycle. However, there was no variability in GAPDH Ct values between treatments, within the same donor samples.

Incubation with 10nM of $1,25(OH)_2D_3$ increased CYP24A1 mRNA expression in both keratinocytes and fibroblasts, under both non-scratched and scratched conditions after 24 hours. Upregulation in CYP24A1 expression was approximately 100-200 times higher in epidermal keratinocytes than dermal fibroblasts. In addition, the upregulatory effect of $1,25(OH)_2D_3$ seen in scratched keratinocytes almost doubled that seen in non-scratched keratinocytes. On the other hand, mRNA expression of VDR, CYP2R1 and CYP27B1 was not modulated by the presence of $1,25(OH)_2D_3$ in either cell types (Table 3-1).

In non-scratched dermal fibroblasts, incubation with 10nM cholecalciferol for 24 hours almost doubled CYP27B1 mRNA expression compared to vehicle control. In scratched fibroblasts, 10nM cholecalciferol increased VDR, CYP2R1, CYP27B1 and CYP24A1 mRNA expression. The increase in CYP24A1 expression was approximately a quarter of that seen in scratched dermal fibroblasts incubated with $1,25(OH)_2D_3$ (Table 3-1).

Incubation with 25(OH)D_3 for 24 hours upregulated CYP24A1 mRNA expression in dermal fibroblasts, at a level lower to that seen in 1,25(OH)$_2$$D_3$. There were no changes to the mRNA expression of VDR, CYP2R1 and CYP27B1 (Table 3-1).

mRNA		Primary keratinocytes	Primary fibroblasts		
		10nM 1,25(OH)$_2$$D_3$	10nM 1,25(OH)$_2$$D_3$	10nM Cholecalciferol	10nM 25(OH)D_3
VDR	**Non-scratched**	0.88 ± 0.09	0.69 ±0.19	0.94 ± 0.05	0.78 ± 0.14
	Scratched	0.99 ± 0.11	0.78 ± 0.11	4.00 ± 0.08 ***	0.83 ± 0.09
CYP2R1	**Non-scratched**	1.03 ± 0.06	0.76 ± 0.12	0.96 ± 0.07	0.88 ± 0.10
	Scratched	0.97 ± 0.08	0.88 ± 0.06	3.53 ± 0.52 ***	0.93 ± 0.05
CYP27B1	**Non-scratched**	1.19 ± 0.13	1.27 ± 0.22	1.81 ± 0.14 **	1.22 ± 0.24
	Scratched	1.10 ± 0.05	1.37 ± 0.24	2.00 ± 0.46 **	1.46 ± 0.26
CYP24A1	**Non-scratched**	1610.55 ± 43.67 ***	15.18 ± 1.64 ***	0.93 ± 0.11	13.55 ± 1.72 ***
	Scratched	2749.59 ± 68.45 ***	12.00 ± 1.38 ***	3.53 ± 0.47 ***	8.55 ± 0.96 ***

Table 3-1: Summary of the relative mRNA expression of VDR, CYP2R1, CYP27B1 and CYP24A1 in primary keratinocyte (n=3) and dermal fibroblast (n=3) after 24h incubation with 10nM of 1,25(OH)$_2$$D_3$ or its precursors. Data presented as the mean relative expression normalised against vehicle control (0.01% ethanol) ± SEM. **Denotes $p<0.01$, *** denotes $p<0.001$ using two-way ANOVA.

3.8 VDR knockdown with siRNA upregulated CYP27B1 mRNA expression, only in scratched dermal fibroblasts.

All dermal fibroblasts were derived from facial skin of female donors with a mean age of 61 years and assayed at passage 3-4 (Table 2-13). All dermal fibroblasts were incubated with or without 10nM $1,25(OH)_2D_3$. Dermal fibroblasts without siRNA transfection were used as a negative control. Relative mRNA expression of VDR, CYP2R1, CYP27B1 and CYP24A1 was normalised against the negative control (i.e. PFSM with 2.5 µl of Lipofectamine® RNAiMAX reagent and 105 µl of opti-MEM).

VDR knockdown in scratched and non-scratched dermal fibroblasts resulted in approximately 89-91% decrease in VDR mRNA expression, in both scratched and non-scratched dermal fibroblasts (Table 3-2). In scratched dermal fibroblasts, there was a 2-fold increase in CYP27B1 expression after silencing of VDR, in the absence or presence of $1,25(OH)_2D_3$. There was no change in CYP27B1 expression in non-scratched dermal fibroblasts (Table 3-2). CYP2R1 and CYP24A1 mRNA expression were not modulated by VDR knockdown (Table 3-2).

mRNA		Vehicle control + non-target siRNA	Vehicle control + VDR siRNA	10nM $1,25(OH)_2D_3$ + non-target siRNA	10nM $1,25(OH)_2D_3$ + VDR siRNA
VDR	Non-scratched	1.10 ± 0.05	0.10 ± 0.01 ***	1.02 ± 0.03	0.11 ± 0.02 ***
	Scratched	0.99 ± 0.04	0.09 ± 0.01 ***	1.01 ± 0.04	0.09 ± 0.01 ***
CYP2R1	Non-scratched	1.04 ± 0.07	0.99 ± 0.03	1.02 ± 0.07	1.03 ± 0.03
	Scratched	1.01 ± 0.05	1.02 ± 0.04	1.03 ± 0.05	1.02 ± 0.05
CYP27B1	Non-scratched	1.02 ± 0.06	1.01 ± 0.03	1.01 ± 0.04	1.10 ± 0.02
	Scratched	1.06 ± 0.05	2.02 ± 0.13 **	0.98 ± 0.03	2.13 ± 0.16 **
CYP24A1	Non-scratched	0.98 ± 0.03	0.97 ± 0.04	1.03 ± 0.05	1.13 ± 0.06
	Scratched	1.04 ± 0.06	1.06 ± 0.06	0.96 ± 0.03	1.08 ± 0.05

Table 3-2: Summary of the relative mRNA expression of VDR, CYP2R1, CYP27B1 and CYP24A1 in human dermal fibroblasts (n=3) after 24h incubation with vehicle control or 10nM of $1,25(OH)_2D_3$ and non-target siRNA/VDR siRNA. Data was presented as the mean relative expression normalised against vehicle control ± SEM. **Denotes $p<0.01$, *** denotes $p<0.001$ using two-way ANOVA test.

3.9 $1,25(OH)_2D_3$ downregulated α-SMA expression in scratched human dermal fibroblasts after 24 hours incubation

It has previously been shown that mechanical scratching increases the expression of α-SMA in cultured human dermal fibroblasts (Pomari *et al*. 2014). To determine whether $1,25(OH)_2D_3$ or its precursors modulate α-SMA expression in dermal fibroblasts, in particular after mechanical scratching, immunofluorescent staining of α-SMA was performed. Dermal fibroblasts were derived from facial skin of three female donors, with a median age of 59 years, all assayed at passage number 3-4 (Table 2-11).

Scratching results in an upregulation of α-SMA expression by dermal fibroblasts in vehicle control (Figure 3-14). However, 10nM $1,25(OH)_2D_3$ significantly downregulated expression of α-SMA in mechanically wounded dermal fibroblasts after 24 hours (Figure 3-15 and Figure 3-16). This downregulation of α-SMA was confirmed by preliminary results using Western Blotting technique (Appendix 9.2). Incubation of scratched dermal fibroblasts with 10nM cholecalciferol and 10nM of 25-hydroxyvitamin D3 for 24 hours had no significant effect on α-SMA protein expression by dermal fibroblasts *in vitro* (Figure 3-15 and Figure 3-16).

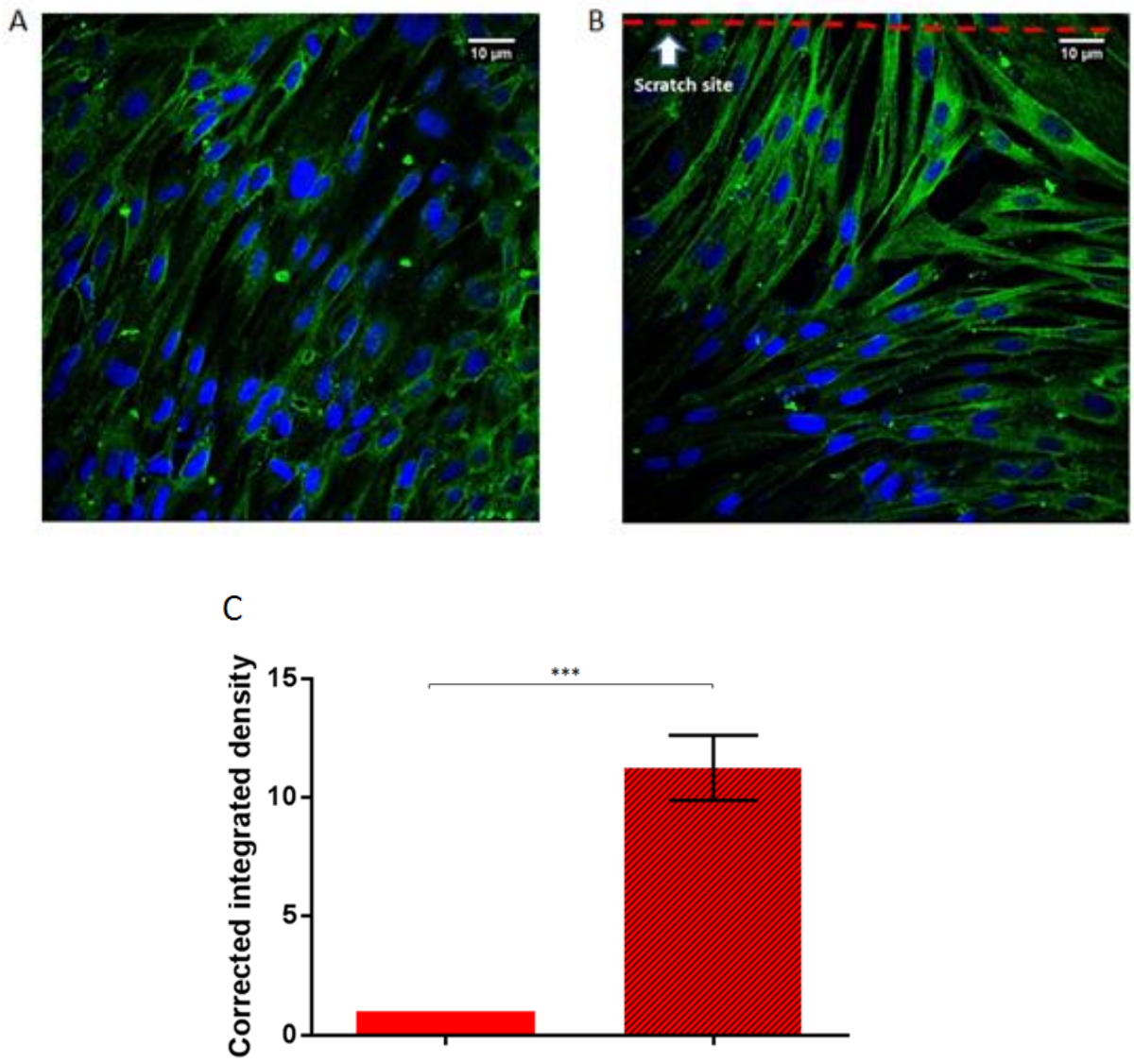


Figure 3-14: Scratching upregulated α-SMA expression in human dermal fibroblasts. α-SMA immunofluorescent staining (green) with nuclear DAPI staining (blue) in (A) non-scratched (unhatched bar) and (B) scratched (hatched bar) dermal fibroblasts. (C) Quantitative data of cytoplasmic staining of α-SMA are presented as patient mean (n=3 donors) ± SEM. ***Denotes $p<0.001$ using paired student's t-test. Magnification = 20x. Scale bar = 10 µm

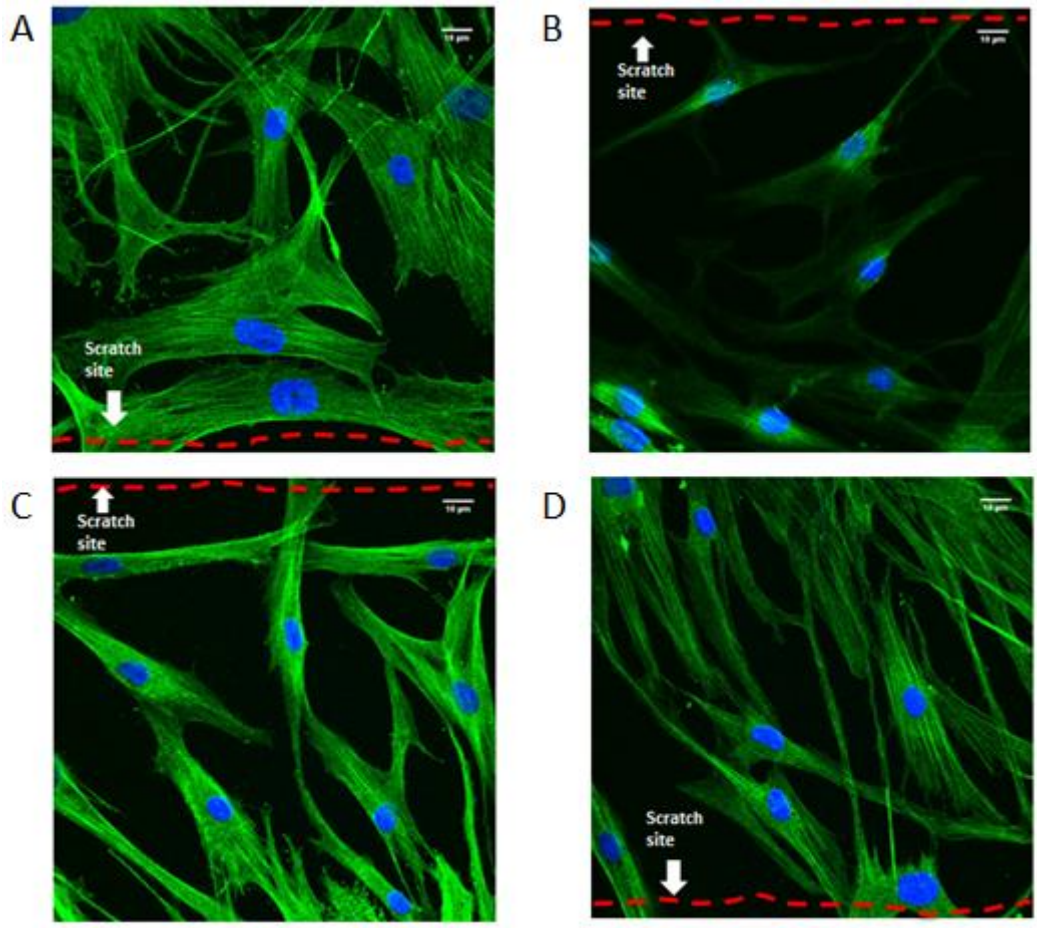


Figure 3-15: Only $1,25(OH)_2D_3$ downregulated α-SMA expression in scratched human dermal fibroblasts. α-SMA immunofluorescent staining (green) with nuclear DAPI staining (blue) in (A) vehicle control (0.01% ethanol); (B) 10nM $1,25(OH)_2D_3$ (C) 10nM cholecalciferol; (D) 10nM $25(OH)D_3$. Magnification = 40x. Scale bar = 10 µm

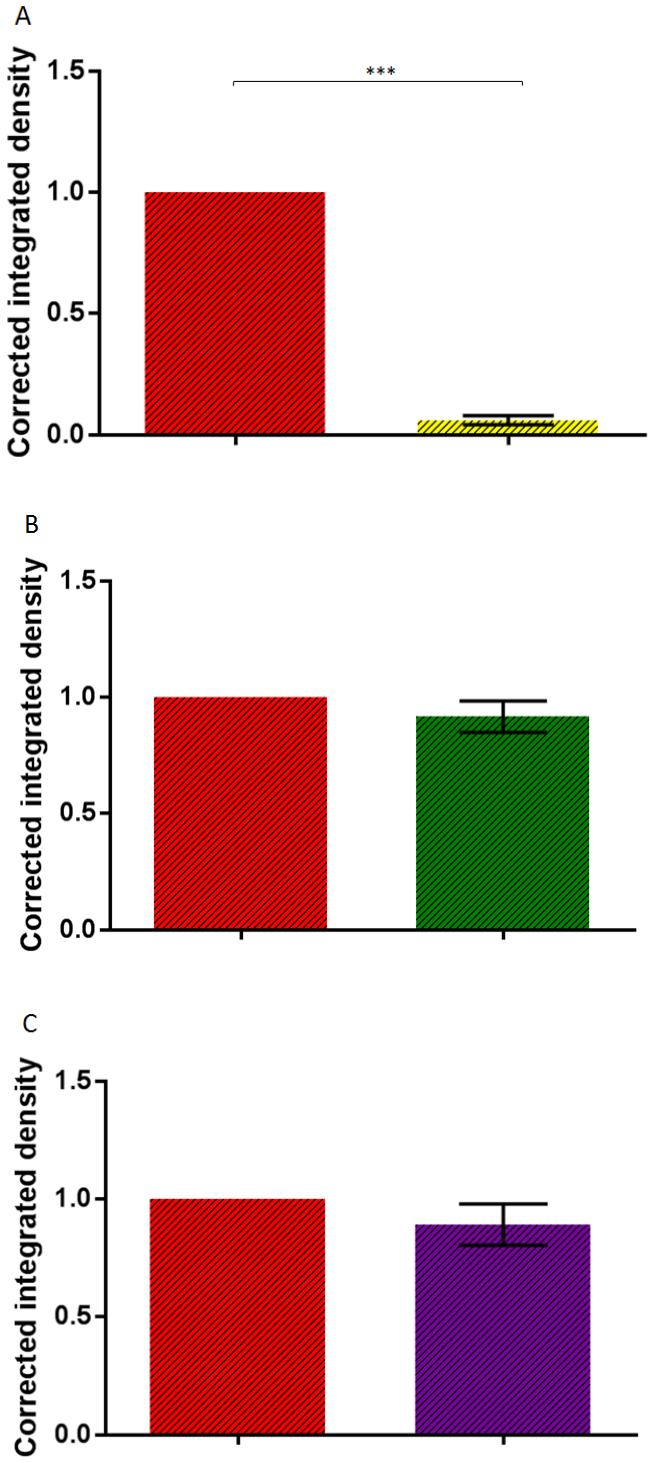


Figure 3-16: Quantitative data of cytoplasmic staining of α-SMA in scratched dermal fibroblasts after 24h incubation with vehicle control-0.01% ethanol (red bars) or (A) 10nM $1,25(OH)_2D_3$ (yellow bars); (B) 10nM cholecalciferol (green bars); and (C) 10nM of $25(OH)D_3$ (purple bars). Data are presented as patient mean (n=3 donors) ± SEM. ***Denotes p<0.001 using paired student's t-test.

3.10 Soluble MMP-2 activity was upregulated by 10nM cholecalciferol but downregulated by 10nM 1,25$(OH)_2D_3$ in scratched dermal fibroblasts after 24 hours

Soluble MMP-2 activity was quantified using gelatine zymography. Dermal fibroblasts were derived from facial skin of three female donors, assayed at passage 3, with a mean age of 62 (Table 2-15).

Scratching cultured dermal fibroblasts resulted in the downregulation of soluble MMP-2 activity (Figure 3-17). Neither 10nM of cholecalciferol or 1,25$(OH)_2D_3$ modulated MMP-2 activity by non-scratched dermal fibroblasts. However, in scratched dermal fibroblasts, cholecalciferol upregulated MMP-2 activity whilst 1,25$(OH)_2D_3$ exert the opposing effect (Figure 3-17).

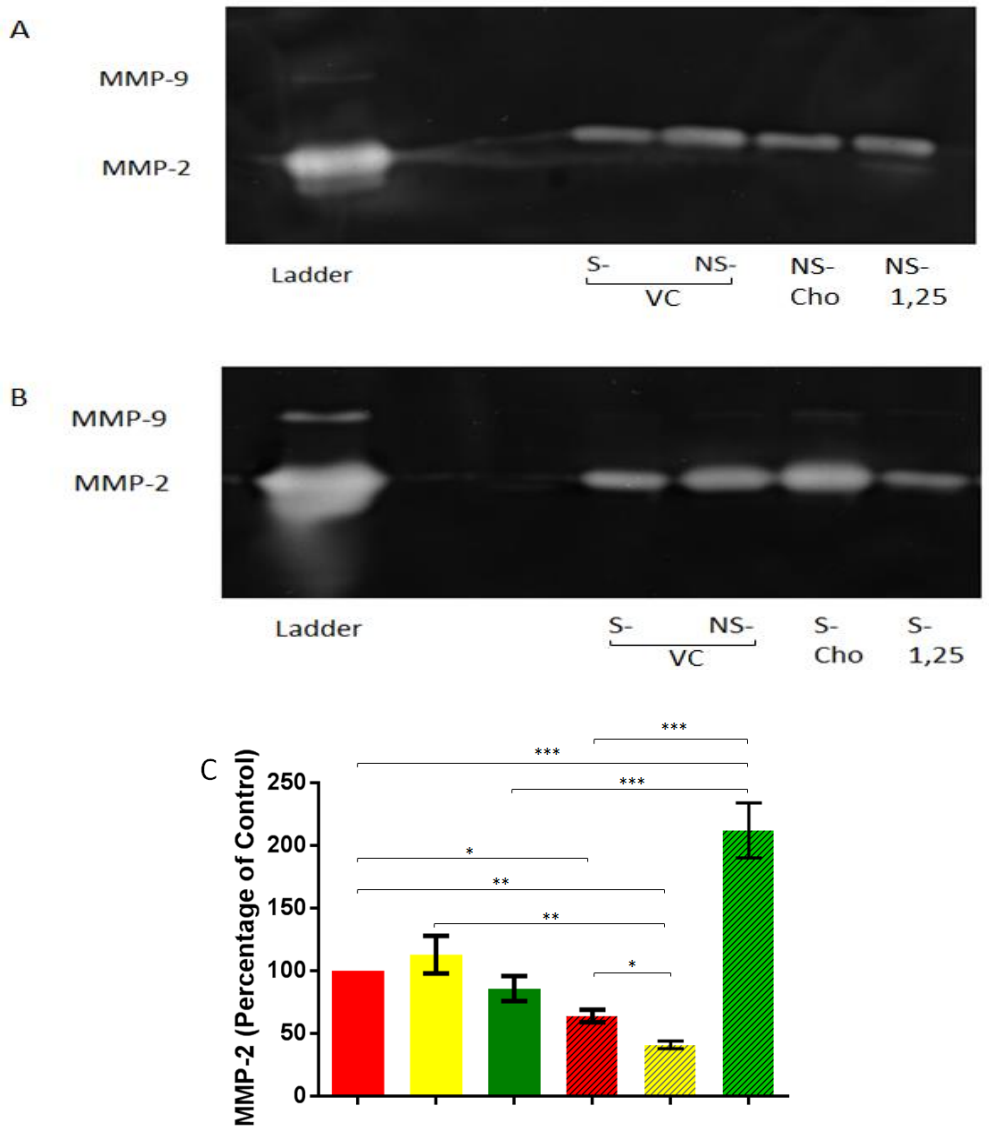


Figure 3-17: Scratching downregulates MMP-2 activity by cultured human dermal fibroblasts. Soluble MMP-2 activity by dermal fibroblasts (non-scratched and scratched) after 24 hours incubation with 10nM cholecalciferol or 10nM $1,25(OH)_2D_3$ were quantified using imageJ software. (A) Gelatine zymography image: S-VC (scratched-vehicle control), NS-VC (non-scratched-vehicle control), NS-Cho (non-scratched, cholecalciferol), NS-1,25 (non-scratched, $1,25(OH)_2D_3$); (B) Gelatine zymography image: S-VC (scratched-vehicle control), NS-VC (non-scratched-vehicle control), S-Cho (scratched, cholecalciferol), S-1,25 (scratched, $1,25(OH)_2D_3$) (C) Data presented as percentage of control (n=3) ± SEM, with non-scratched dermal fibroblasts (vehicle control) being the control. Non-scratched dermal fibroblasts (unhatched bars); scratched dermal fibroblasts (hatched bars); vehicle control (0.01% ethanol) – red bars; 10nM $1,25(OH)_2D_3$ – yellow bars; 10nM cholecalciferol – green bars *Denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$ using two-way ANOVA.

3.11 $1,25(OH)_2D_3$ upregulated type I collagen mRNA expression in non-scratched dermal fibroblasts. However, collagen I/III mRNA expression and type I to III collagen ratio were downregulated by $1,25(OH)_2D_3$ in scratched dermal fibroblasts.

The effect of $1,25(OH)_2D_3$ on mRNA expression of collagen I and collagen III by cultured dermal fibroblasts were assessed using qRT-PCR. Mechanical scratching increased mRNA expression of type I collagen while having no effect on type III collagen expression at 24 hours (Figure 3-18). Type I to III collagen ratio was upregulated (Figure 3-18).

In non-scratched dermal fibroblasts, incubation with 10nM and 100nM of $1,25(OH)_2D_3$ upregulated collagen I mRNA expression. The opposite effect was observed in scratched dermal fibroblasts, where incubation with 1nM, 10nM and 100nM of $1,25(OH)_2D_3$ downregulated both collagen I and collagen III mRNA expression.

24 hours incubation with 10 and 100nM of $1,25(OH)_2D_3$ upregulated type I to III collagen ratio in non-scratched dermal fibroblasts. In scratched fibroblasts, type I to III collagen ratio was downregulated by $1,25(OH)_2D_3$ at all concentrations (Figure 3-19).

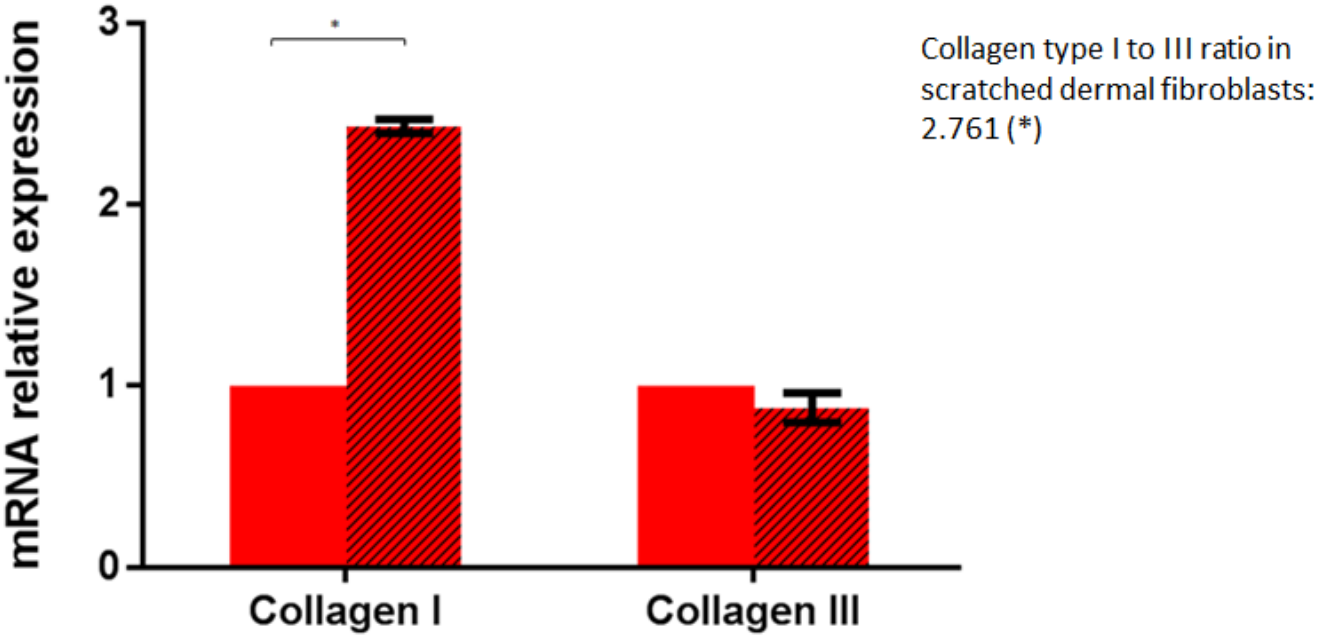


Figure 3-18: Scratching upregulates collagen I mRNA expression in cultured human dermal fibroblasts. mRNA expression was normalised against non-scratched dermal fibroblasts. Data was presented as the mean of 3 donors ± SEM. Non-scratched dermal fibroblasts (unhatched bars); scratched dermal fibroblasts (hatched bars); *Denotes $p<0.05$ using paired student's t-test.

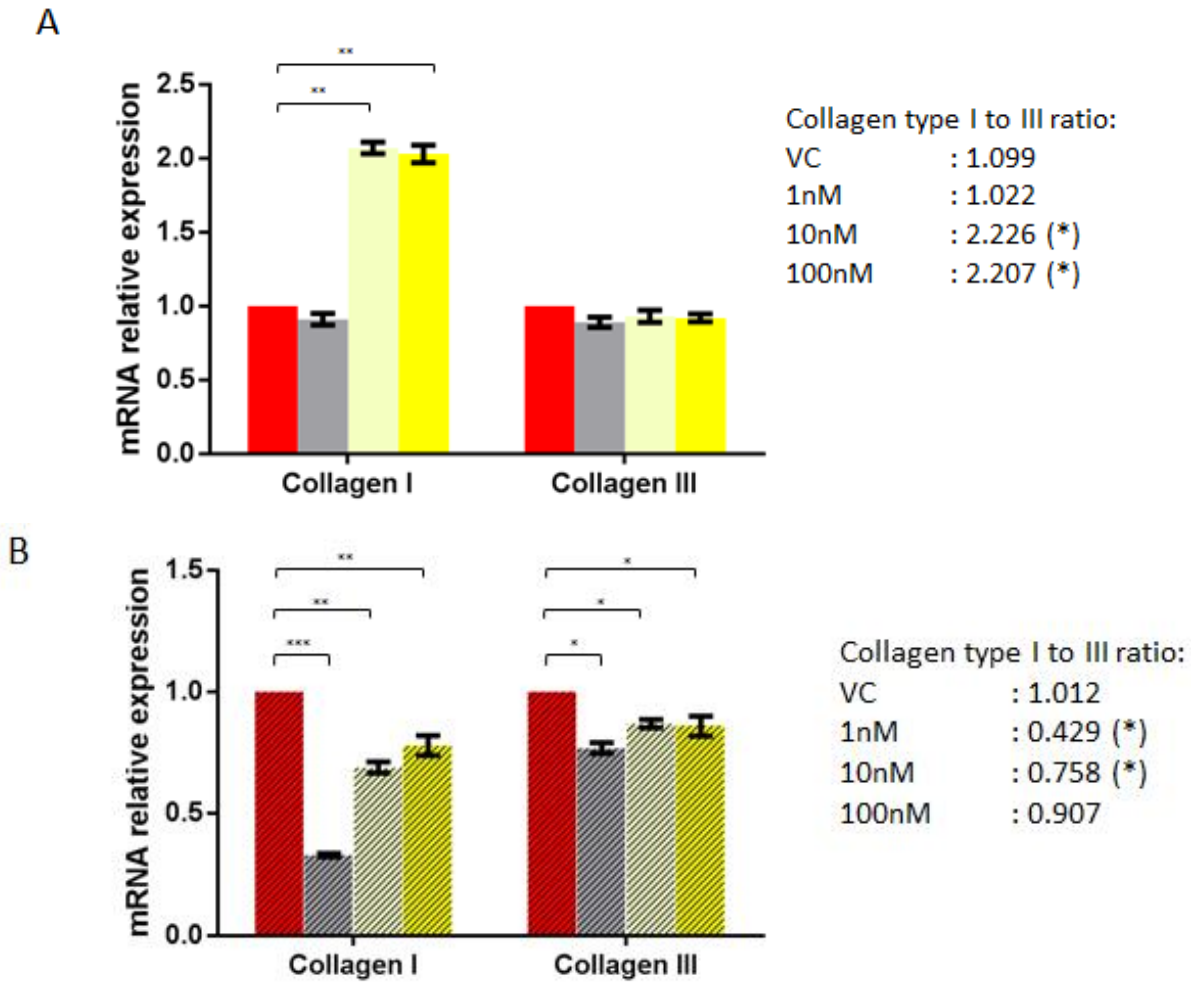


Figure 3-19: Collagen I and collagen III mRNA expression in the presence or absence of $1,25(OH)_2D_3$. mRNA expression was normalised against vehicle control (0.01% ethanol) in either (A) non-scratched (unhatched bars) or (B) scratched (hatched bars) dermal fibroblasts. Data was presented as the mean of three donors ± SEM. Vehicle control (red bars); 1nM $1,25(OH)_2D_3$ (grey bars); 10nM $1,25(OH)_2D_3$ (light yellow); 100nM $1,25(OH)_2D_3$ (dark yellow); *Denotes $p<0.05$, **Denotes $p<0.01$, ***Denotes $p<0.001$ using two-way ANOVA.

3.12 The establishment of an e*x vivo* human skin wound healing model

Figure 3-20 showed that partial thickness wounds artificially created on skin explants gradually closed over a 6 day period. Haematoxylin and Eosin (H&E) staining demonstrated the extension of the epithelial tongue into the wound at day 6. This is characterised by epithelial cells from the epidermis neighbouring the wound site migrate over the surface of the wound bed. There was an increase in the thickness of granulation tissues at day 6 compared to immediately after wounding (Figure 3-20).

The effect of cholecalciferol or $1,25(OH)_2D_3$ on cutaneous wound repair was assessed by measuring the rate of wound closure (re-epithelialization) at different time points.

Two separate *ex vivo* wound healing studies were carried out using either abdominal skin (female donor, age 59) or breast skin (female, age 34) (See Table 2-16).

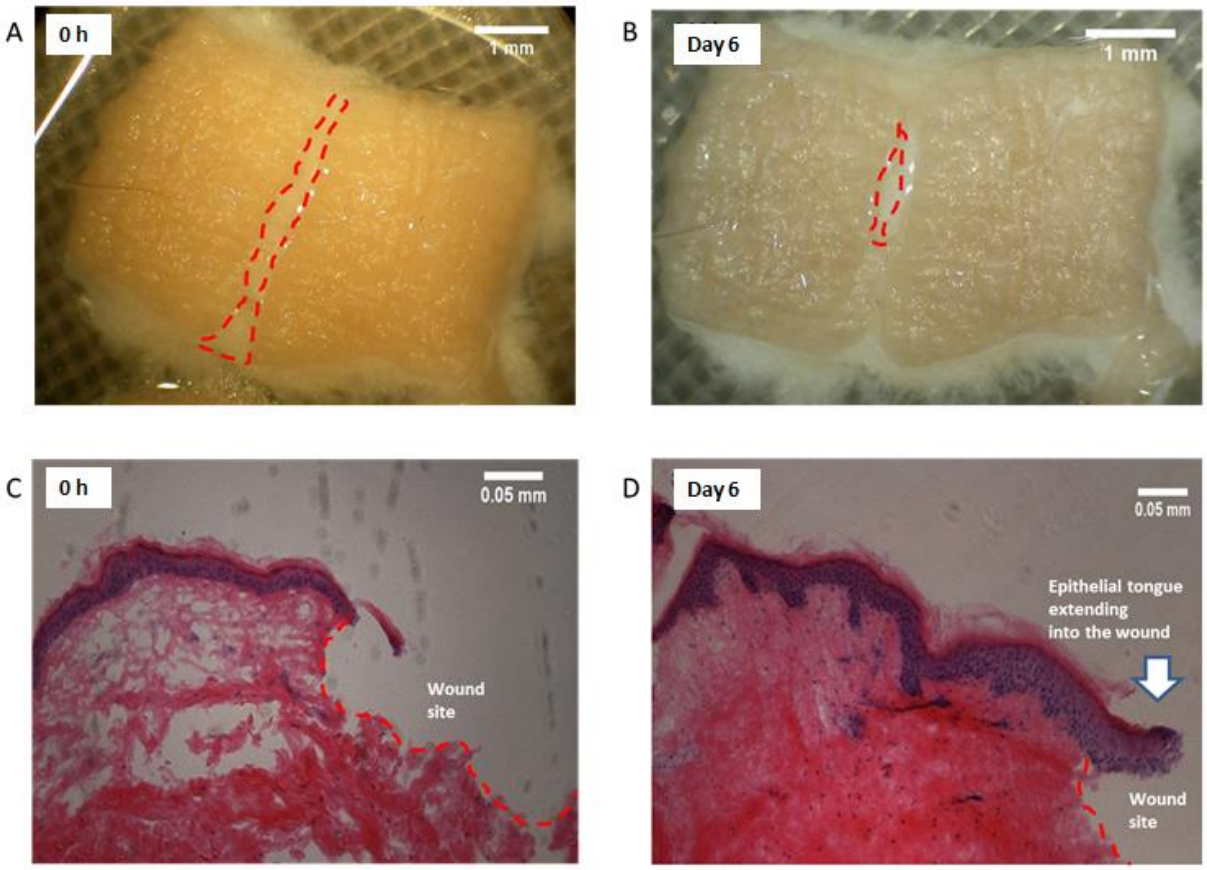


Figure 3-20: Human skin explants used for e*x vivo* wound healing model. A partial thickness incisional wound was created, comprising the epidermis and upper part of the dermis, by making two parallel incision with a scalpel. The width of each cut was approximately 1mm. (A) Partial thickness incisional wound at 0 hour. (B) Partial thickness incisional wound after 6 days. (C) H&E staining of representative section of 8 µm, cut nearest to the center of the wound at 0 hour. (D) H&E staining of representative section of 8 µm, cut nearest to the center of the wound after 6 days. There was an increase in the thickness of the granulation tissue at day 6 compared to immediately after wounding. Wounded area marked with red dotted line.

3.12.1 1,25(OH)$_2$D$_3$, but not cholecalciferol accelerated rate of wound closure in a human *ex vivo* model.

In abdominal skin, incubation with 10% FBS increased the rate of wound closure after 3 days (n=3 replicates). Incubation with 10nM 1,25(OH)$_2$D$_3$ produced similar upregulation at day 3, independent to the presence of FBS. Incubation with both 10% FBS and 10nM 1,25(OH)$_2$D$_3$ did not increase the rate of wound closure any further. At day 6, there was no difference in wound closure rate between the groups (Figure 3-21).

Ex vivo wound healing assay was repeated using breast skin, with an increased number of replicates of group. After 4 days, 10% FBS and 10nM 1,25(OH)$_2$D$_3$ both increased the rate of wound closure. The effect of FBS (240%) was greater than 1,25(OH)$_2$D$_3$ (115%), although the difference between the two groups was not statistically significant. Cholecalciferol had no effect as seen previously. After 6 days, while 1,25(OH)$_2$D$_3$ had no effect as seen in the previous donor, FBS still exerted a stimulating effect on wound closure by 166% (Figure 3-22).

The third *ex vivo* wound healing assay was performed using abdominal skin again, with a higher number of technical replicates per treatment group this time. 10% FBS and 10nM 1,25(OH)$_2$D$_3$ both increased the rate of wound closure after 3 days (n=8 replicates). At day 6, only 10% FBS produced an increase in wound closure (n=5 replicates) but no effect was seen with either cholecalciferol or 1,25(OH)$_2$D$_3$ (Figure 3-23).

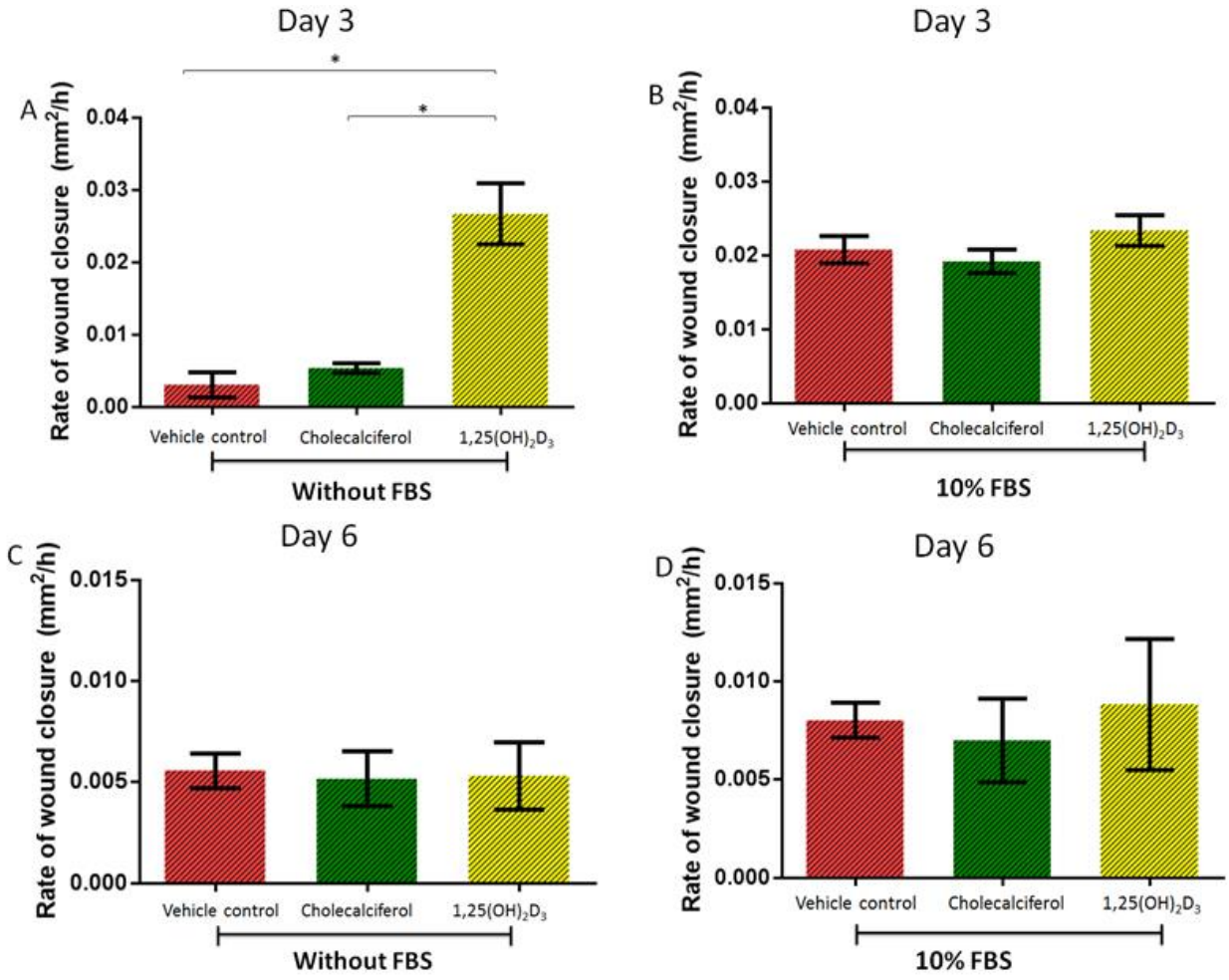


Figure 3-21: Rate of closure of *ex vivo* wounds (abdominal skin) treated with 10nM cholecalciferol or 10nM $1,25(OH)_3D_3$ with or without FBS. Negative control refers to PFSM with 0.01% ethanol and positive control refers to PFSM with 10% FBS. (A) Rate of wound closure at day 3 without FBS; (B) Rate of wound closure at day 3 with 10% FBS; (C) Rate of wound closure at day 6 without FBS; (D) Rate of wound closure at day 6 with 10% FBS. n=3 technical replicates per group at 3 days after wounding and n=2 technical replicates per group at 6 days hours after wounding. Vehicle control (red bars); 10nM cholecalciferol (green bars); 10nM $1,25(OH)_2D_3$ (yellow bars); *Denotes $p<0.05$ using one-way ANOVA.

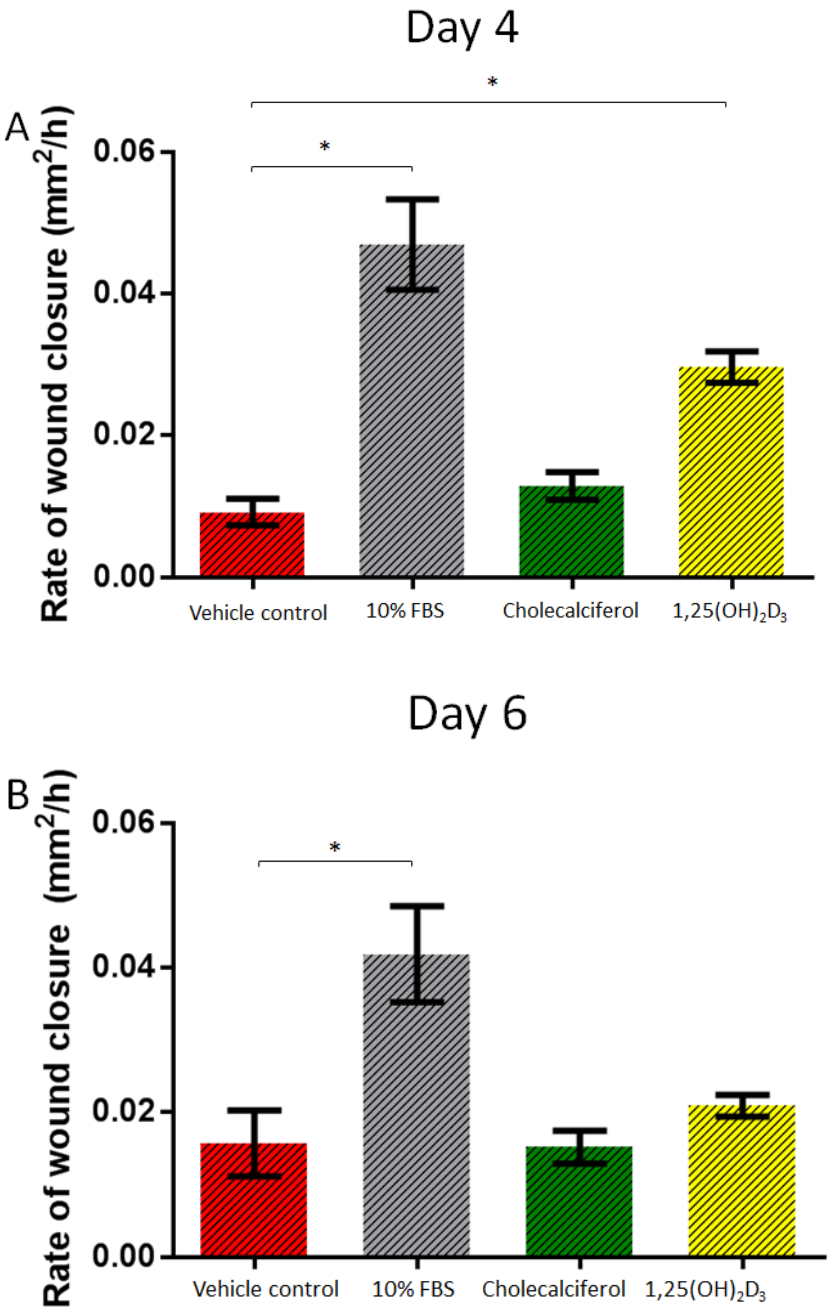


Figure 3-22: Rate of closure of *ex vivo* wounds (breast skin) treated with 10nM cholecalciferol or 10nM 1,25(OH)$_3$D$_3$. PFSM with 0.01% ethanol was used as a negative control and PFSM with 10% FBS was used as a positive control. (A) Rate of wound closure at 4 days; (B) Rate of wound closure at 6 days. n=8 technical replicates per group at day 4 after wounding and n=5 technical replicates per group at day 6 after wounding. Serum free media (red bars); PFSM with 10% FBS (grey bars); PFSM with 10nM cholecalciferol (green bars); PFSM with 10nM 1,25(OH)$_2$D$_3$ (yellow bars). *Denotes $p<0.05$ using one-way ANOVA.

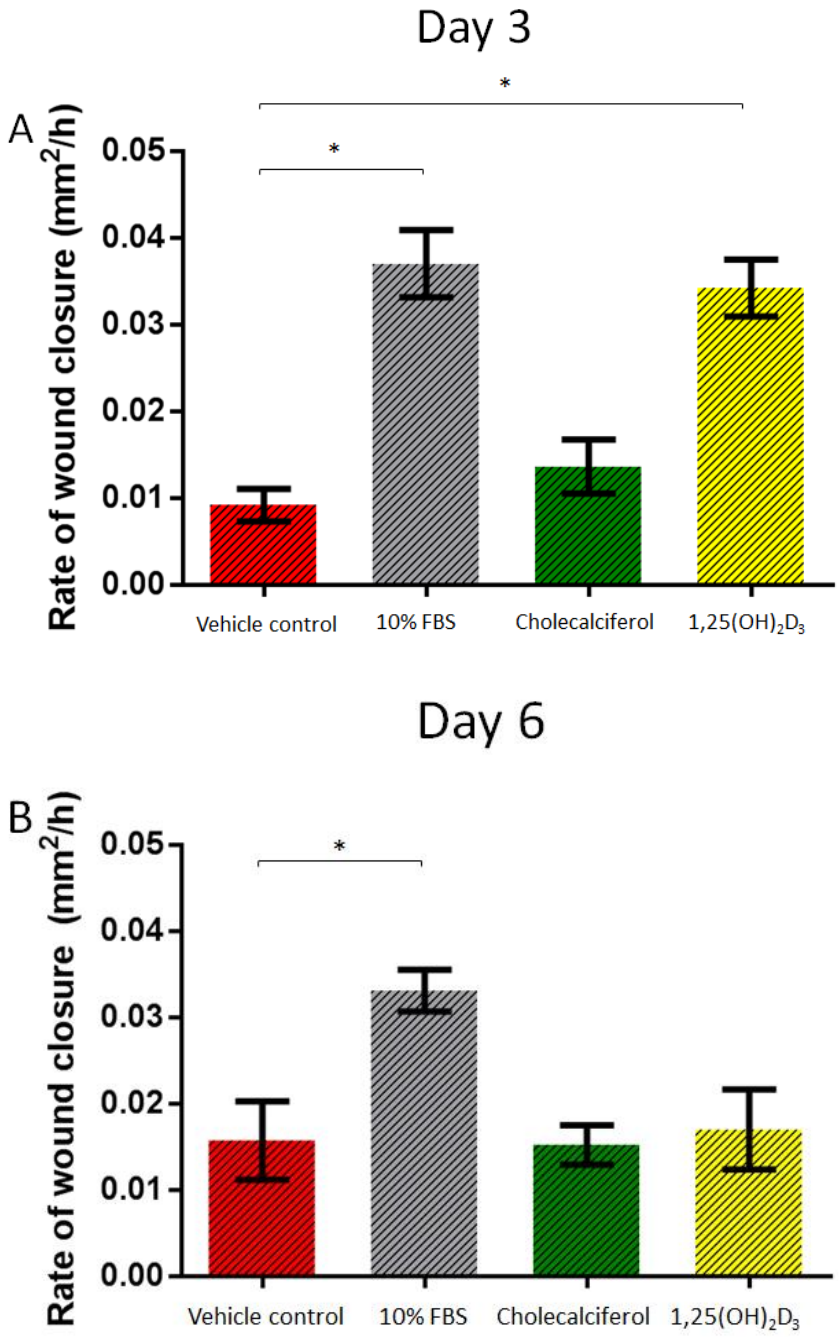


Figure 3-23: Rate of closure of *ex vivo* wounds (abdominal skin) treated with 10nM cholecalciferol or 10nM 1,25(OH)$_3$D$_3$. PFSM with 0.01% ethanol was used as a negative control and PFSM with 10% FBS was used as a positive control. (A) Rate of wound closure at 3 days; (B) Rate of wound closure at 6 days. n=8 technical replicates per group at day 3 after wounding and n=5 technical replicates per group at day 6 after wounding. Serum free media (red bars); PFSM with 10% FBS (grey bars); PFSM with 10nM cholecalciferol (green bars); PFSM with 10nM 1,25(OH)$_2$D$_3$ (yellow bars). *Denotes $p<0.05$ using one-way ANOVA.

4 DISCUSSION

The ageing population presents a huge challenge to the healthcare service. As the elderly is commonly burdened with chronic non-healing wounds, new therapies aimed at improving wound healing are always being sought. Over the past decades, there is an increasing recognition in the multitude of regulatory roles played by vitamin D in maintaining healthy tissue functions. Low serum level of vitamin D is associated with chronic non-healing venous ulcers in clinical trials (Burkiewicz et al. 2012). In addition, vitamin D supplementation for 12 weeks was shown to improve wound healing in patients suffering from diabetic foot ulcer (Razzaghi *et al*. 2017). In murine study, absence of VDR or $1,25(OH)_2D_3$ resulted in impaired granulation tissue formation (Luderer *et al*. 2013). While there is renewed interest in exploring the molecular pathways that vitamin D utilises to exert its regulatory functions in the skin, most studies have placed emphasis on the epidermal keratinocyte model (Vantieghem *et al*. 2006; Gombart 2009). Little is known about the roles of vitamin D in human dermal fibroblasts. In addition, many questions remain unanswered about the autocrine and paracrine functions served by dermal fibroblasts in regulating the bioavailability of active vitamin D in the skin.

4.1 Dermal fibroblast express higher VDR mRNA while epidermal keratinocytes express higher CYPs

Previous studies have shown that a complete and functional photo-endocrine vitamin D system exists in human epidermal keratinocytes (Vantieghem *et al*. 2006; Bikle 2011). Upon UVB irradiation, epidermal keratinocytes are able to produce cholecalciferol from endogenous 7-dehydrocholesterol (Vantieghem *et al*. 2006). Cholecalciferol is further activated in the skin via a two-step hydroxylation process to the physiologically active form, $1,25(OH)_2D_3$ (Vantieghem *et al*. 2006). Although there have been previous studies confirming the expression of VDR in the human dermal fibroblast, there is limited, conflicting literature providing evidence to dermal vitamin D_3

photoproduction (Vantieghem *et al*. 2006; Ellfolk *et al*. 2009; Zerr *et al*. 2015). A recent study by Norlin *et al* (2017) suggested that primary dermal fibroblasts also have an active vitamin D_3 metabolising system and express mRNA for VDR (Norlin et al. 2017). In their study, 25-hydroxylase (CYP2R1) and 1α-hydroxylase (CYP27B1) activities in cultured human dermal fibroblasts were confirmed using high performance liquid chromatography (HPLC) and an enzyme-linked immunosorbent assay (ELISA) (Norlin et al. 2017). The limitation in their study is that it was not specified in the study which body sites the skin samples were harvested from, although the study did mention that it was skin donated from plastic surgery excision biopsy. In another study by Vantieghem *et al* (2006) using dermal fibroblasts derived from juvenile foreskin, it was reported that only CYP2R1 and CYP24A1 activity were detected using Northern Blot analysis, but CYP27B1 was not detected (Vantieghem *et al*. 2006).

In order to clarify, the present study uses both donor-matched (n=3 donors) and non donor-matched keratinocytes (n=3 donors) and fibroblasts (n=8 donors). It has been demonstrated that both epidermal keratinocyte and dermal fibroblast exhibit a complete vitamin D_3 metabolising system at the transcriptional level (Figure 3-1 and Figure 3-2). Moreover, by using donor-matched samples, it was shown for the first time that human dermal fibroblast expressed a much higher level of VDR than keratinocytes (Figure 3-1 and Figure 3-2). This suggests that fibroblasts could be more responsive to the actions of $1,25(OH)_2D_3$ than keratinocytes. In addition, by including non donor-matched samples, data from up to eight individual donors were analysed to account for possible inter-individual variability. The finding that VDR mRNA expression was still significantly higher in fibroblasts lends strength to the observation in donor-matched samples. Higher VDR expression by dermal fibroblast indicates that fibroblast is an active key player in regulating normal tissue homeostasis in the skin. This is in contrast to the traditional view of fibroblast as a quiescent cell solely responsible for extracellular matrix production (Frazier *et al*. 1996; Roh *et al*. 2010). While epidermal keratinocytes play a central role in maintaining the integrity of epithelial barrier, dermal

fibroblast has a wider range of functions, due to its close proximity and cross-talk with other cell types such as adipocytes, vascular endothelial cells and inflammatory cells (Hughes 2008; Ezure and Amano 2011; Van Linthout *et al*. 2014; Costa-Almeida *et al*. 2015; Haubner *et al*. 2015).

In contrast cultured human epidermal keratinocytes expressed higher levels of the vitamin D_3 metabolising enzymes – CYP2R1, CYP27B1 and CYP24A1 than dermal fibroblasts in both donor-matched and non donor-matched samples. This is hardly surprising as the highest concentration of 7-dehydrocholesterol (7-DHC) is found in the epidermis, mainly in the *stratum basale* and *stratum spinosum* (Gaylor and Sault 1964; Herrmann *et al*. 2016). 7-DHC is an intermediate metabolite during endogenous synthesis of cholecalciferol. It is secreted by the sebaceous glands and absorbs UVB radiation most effectively at wavelengths of 280-315 nm for photolysis of the B ring of the sterol nucleus (Whiting and Calvo 2013). UVB with a relatively high frequency and short wavelength reaches the skin and is absorbed to a great extent by the epidermis (Figure 4-1). Only a small fraction of UVB reaches the dermis (Gupta *et al*. 2013). The anatomical positioning of keratinocytes and fibroblasts within different layers of the skin hence plays a large part in determining their participation in the photoproduction of active vitamin D_3. This could also partly explain the conflicting findings by Vantieghem *et al* (2006) and Norlin *et al* (2017). Skin from juvenile foreskin and plastic surgery skin biopsy were used respectively in each study. Juvenile foreskin is generally non sun-exposed. In contrast, skin donated in plastic surgery is commonly obtained from body sites that has greater sun exposure such as face and neck (Trost et al. 2016). This present study has used skin from facelift surgery (Table 2-1), and it showed that dermal fibroblast expressed the VDR and all the CYPs at the transcriptional level (Figure 3-1 and Figure 3-2), which correlates with the findings by Norlin *et al* (2017). This supports the hypothesis that there is site specific variation in the activity of the CYPs in the dermis, and only sun-exposed dermal fibroblasts possesses the complete vitamin D_3 metabolism machinery.

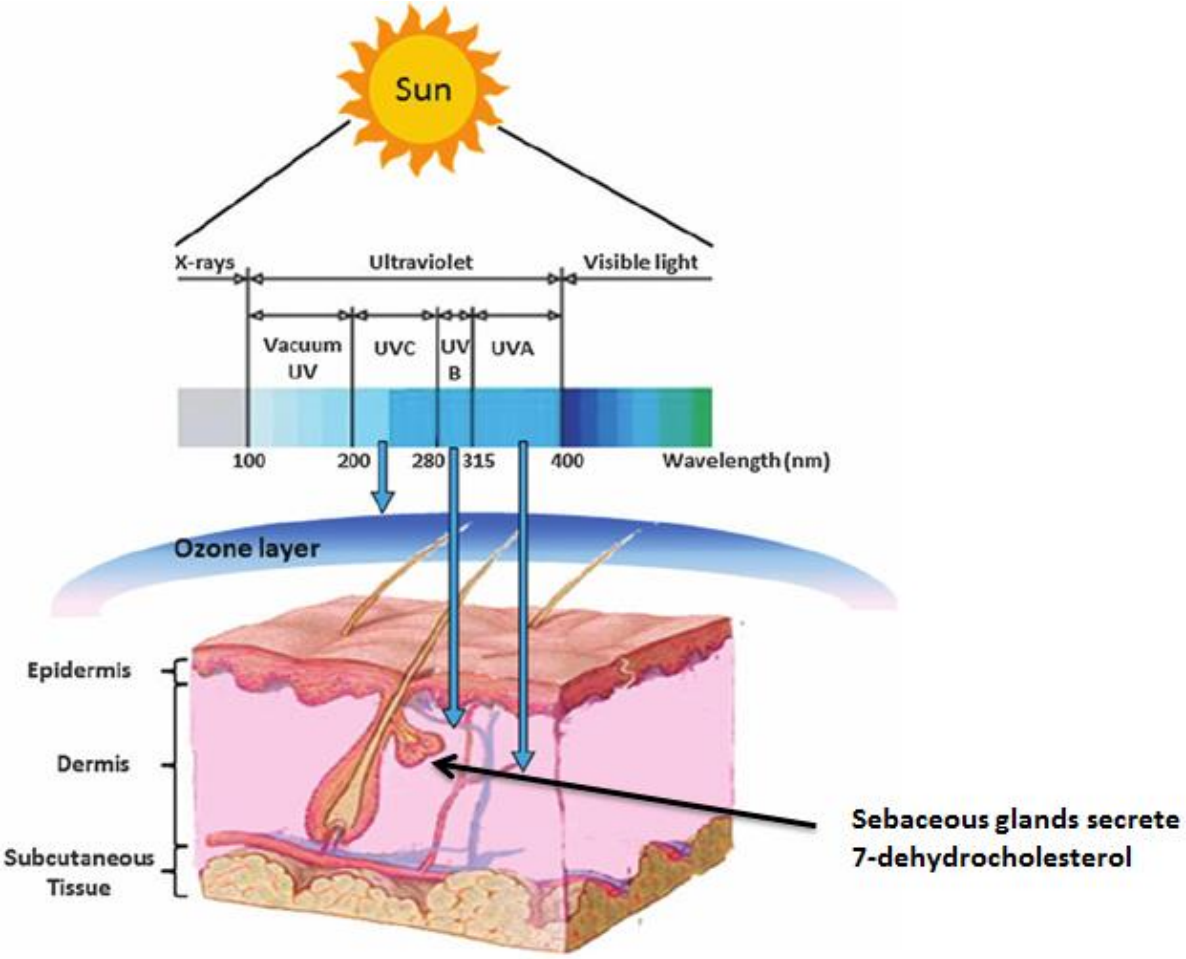


Figure 4-1: **Wavelength-dependent penetration of UV radiation in the skin affects site of vitamin D_3 photoproduction.** UVB of 280-315nm wavelengths are absorbed to a greater extent in the epidermis. Sebaceous glands secrete 7-dehydrocholesterol, an intermediate metabolite in the synthesis of cholecalciferol in the skin. It then accumulates at the Malpighian layer of the epidermis, in the stratum spinosum and stratum basale (Beckman and Deluca 1998). Adapted from (Gupta et al. 2013)

4.2 $1,25(OH)_2D_3$ stimulates epidermal keratinocyte migration. Conversely, it inhibits dermal fibroblast migration in a scratch wound assay.

Keratinocyte migration is an important part of the re-epithelialization process during wound healing. For keratinocyte migration to occur, the cells first needs to detach from the underlying basal lamina, a process dependent on loss of cell-cell and cell-matrix adhesion (Wu et al. 2016). Previous studies using HaCaT keratinocyte cell line has shown an increase in migration following incubation with $1,25(OH)_2D_3$ (Adiri 2009). In agreement with the results from cell line studies, this study showed that $1,25(OH)_2D_3$ at 1, 10 and 100nM upregulates human epidermal keratinocyte migration at 24 hours (Figure 3-3). In contrast, $1,25(OH)_2D_3$ inhibits dermal fibroblast migration at all three concentrations, as early as 4 hour and for up to 24 hours (Figure 3-5).

Effect seen after 24 hours – the genomic response

$1,25(OH)_2D_3$ increases keratinocyte migration

An increase in keratinocyte migration is possibly to be mediated by steroid receptor coactivator (Src) (Rotty and Coulombe 2012; Sophors et al. 2016; Wu et al. 2016). Being a coactivator, it is known that Src protein interacts with the activation domain, AF-2 on VDR to augment $1,25(OH)_2D_3$-dependent transcription (Gill et al. 1998; Takeyama et al. 1999; Buitrago et al. 2000; Zhang et al. 2001; Choi et al. 2011). Once $1,25(OH)_2D_3$ binds to VDR, it induces a conformational change of AF-2, thus allowing protein-protein contacts between the VDR and Src to form a ternary complex (Masuyama et al. 1997). The bound Src then functions as bridging proteins that link VDR to RNA polymerase II and the basal transcription machinery (Zhang et al. 2001). In addition, Src also interacts with general transcription factors (transcription factor IIB/TATA-binding protein) and general transcription activator (CBP/p300) to form a multi-protein complex assembled at the VDRE of targeted gene promoters (Kamei et al. 1996; Takeshita et al. 1996; Yao et al. 1996; Zhang et al. 2001). The intrinsic

histone acetyltransferase activity of Src also helps to remodel chromatin structure to allow VDR-RXR heterodimers greater access to the DNA transcriptional machinery (Zhang et al. 2001; Deeb et al. 2007; Carlberg and Seuter 2009; Carlberg and Campbell 2013).

A recent *in vitro* study by Wu *et al* (2016) using human epidermal keratinocytes showed that Src is important in upregulating MMP-2 secretion and downregulates E-cadherin, which enhanced a transient breakdown of extracellular matrix and cell-cell adhesion to initiate keratinocyte migration (Wu et al. 2016). Src overexpression promotes keratinocyte migration and Src silencing shows the opposite effect (Wu et al. 2016). Downregulation of E-cadherin is also an important marker for epithelial to mesenchymal transition (EMT). Loss of apical-basal polarity of keratinocytes allows rearrangement of cytoskeletal structures for enhanced keratinocyte motility, more closely resembling the mesenchymal features (Levayer and Lecuit 2008; Yan et al. 2010; Micallef et al. 2012; Lamouille et al. 2014). As $1,25(OH)_2D_3$ is capable of interacting with the Src protein via binding to VDR, it can be postulated that MMP-2 and E-cadherin genes are important downstream target gene for $1,25(OH)_2D_3$ to exert its modulatory effect on keratinocyte migration (Figure 4-2) (Masuyama et al. 1997; Yamauchi et al. 2012). In this study, the observed increase in keratinocyte migration was only significant at 24 hours. This late response suggests that a genomic response was required for $1,25(OH)_2D_3$ to exert its migratory effect in keratinocyte (Figure 3-3).

$1,25(OH)_2D_3$ decreases fibroblast migration after 24 hours

Paradoxically, dermal fibroblast migration was inhibited by $1,25(OH)_2D_3$ (Figure 3-4 and Figure 3-5). The observed difference as compared to epidermal keratinocytes indicates that response to $1,25(OH)_2D_3$ are cell-type specific in the skin. Binding of $1,25(OH)_2D_3$ to VDR allows its partner receptor, RXR, to bind the corepressors such as nuclear receptor corepressor (NcoR) or silencing mediator of retinoid and thyroid hormone receptor (SMRT) (Sánchez-Martínez et al. 2008). Binding of 9-cis RA to RXR causes dissociation of these

corepressor from the VDR-RXR heterodimer (Figure 4-2) (Kang et al. 1997; Sanchez-Martinez et al. 2008). Studies have shown that human dermal fibroblast expresses higher level of retinoic acid receptor (RAR)α than epidermal keratinocyte (Redfern and Todd 1992). As 9-cis retinoic acid (9-cis RA) is a ligand to both RAR and RXR, it is possibly that in dermal fibroblast, when both VDR and RARα are highly expressed, there is reduced local bioavailability of endogenous 9-cis RA to bind to RXR. This leads to a reduction in the release of co-repressor from the 1,25$(OH)_2D_3$-activated VDR-RXR heterodimer, resulting in downregulation of the transcription of downstream target genes such as MMP-2. This manifests as an inhibition in dermal fibroblast migration (Figure 3-5).

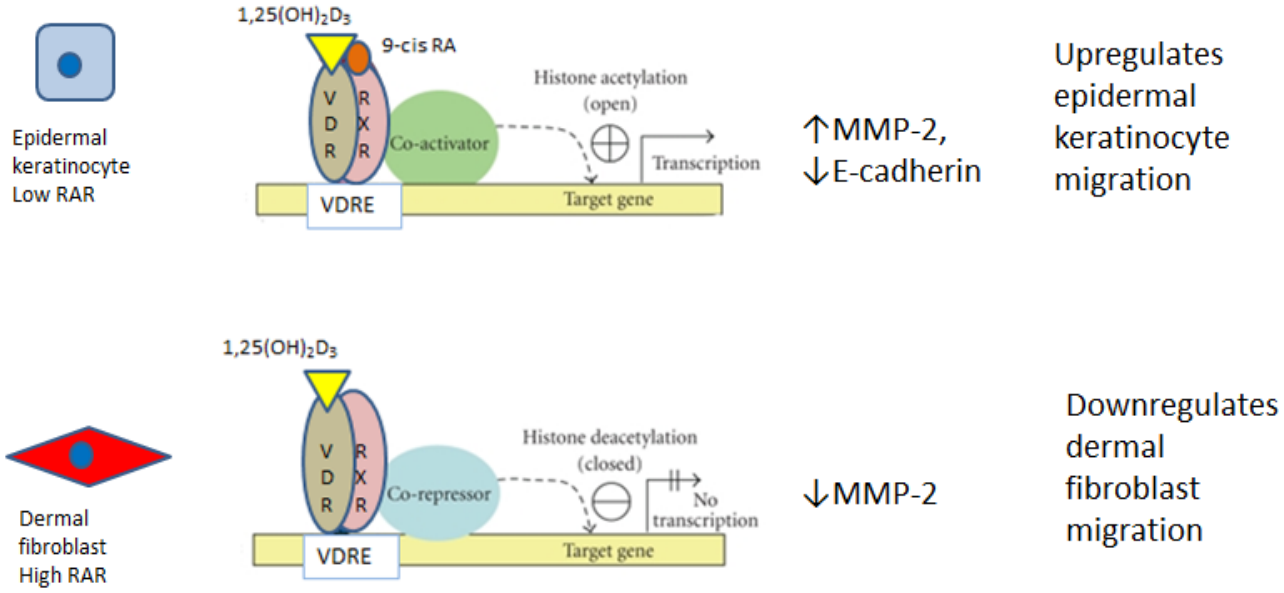


Figure 4-2: Dermal fibroblast expresses higher level of RAR, which compete for the binding of 9-cis RA and reduces bioavailability of endogenous 9-cis RA. Without 9-cis RA binding, co-repressor is bound to the activated VDR-RXR complex, thereby downregulates transcription of downstream target genes such as MMP-2. Adapted from (Kim 2008).

Effect seen as early as 4 hours – the non-genomic response

Early inhibition of dermal fibroblast migration is possibly to be mediated by MARRS and calcium channel modulation

Upon wounding, fibroblast migrates into the wound site and differentiates into myofibroblast, to exert contraction at wound area (Kohyama et al. 2008). However, excessive fibroblast migration into the wound area can distort normal parenchymal elements of tissues, resulting in excessive tissue remodelling and fibrotic changes (Kohyama et al. 2008; Zhao et al. 2016). In the present study, $1,25(OH)_2D_3$ inhibits dermal fibroblast migration as early as 4 hours. The early response can be explained by the non-genomic actions of $1,25(OH)_2D_3$ (Buitrago et al. 2001; Trochoutsou et al. 2015). A rapid non-genomic response of $1,25(OH)_2D_3$ has been described in the modulation of rat aortic vascular smooth muscle cell migration (Rebsamen et al. 2002). The rapid inhibitory effect on fibroblast migration is achieved through the activation of PI3K/Akt/GSK3β/β-catenin pathway (Figure 4-3) (Imai and Clemmons 1999; Reiske et al. 1999; Vanhaesebroeck et al. 1999; Rebsamen et al. 2002; Lam et al. 2011). β-catenin is important in regulating cell migration and adhesion (Amini-Nik et al. 2014). Once $1,25(OH)_2D_3$ binds to MARRS on dermal fibroblasts, it activates PI3K/Akt/GSK3β/β-catenin signalling in the cytosol, leading to an accumulation of cytosolic β-catenin, with reduced nuclear β-catenin levels. This is accompanied by a reduction in the level of MMP-2 and MMP-9 (downstream targets of β-catenin) and result in a decrease in dermal fibroblast motility (Bian et al. 2000; Richard et al. 2010; Vaid et al. 2011; Webb et al. 2017).

Another possible non-genomic pathway that could have been employed involves the downregulation of calcium channels (Yang and Huang 2005). A study by Yang *et al* (2005) showed that calcium influx via voltage-gated calcium channel into mouse embryonic fibroblasts increases fibroblast migration via phosphorylation of the myosin light chain at the trailing end of the migrating fibroblast (Yang and Huang 2005). In the present study, the calcium concentration used in the media contains 1.8 mM of calcium, a level sufficient

for normal cellular function of human dermal fibroblast (Praeger and Gilchrest 1986). It has been shown in previous studies that $1,25(OH)_2D_3$ at a concentrations of 1nM to 100nM can downregulate L-type voltage sensitive calcium channels in neuron cells (Brewer et al. 2001; Gezen-Ak et al. 2011). Hence, it is possibly that $1,25(OH)_2D_3$ decreases motility of dermal fibroblast by reducing the expression of the apical membrane calcium channel at the brush border of the fibroblast cellular membrane, thereby causing a decrease in activation of the myosin light chain in fibroblasts, manifested as an inhibition of migration.

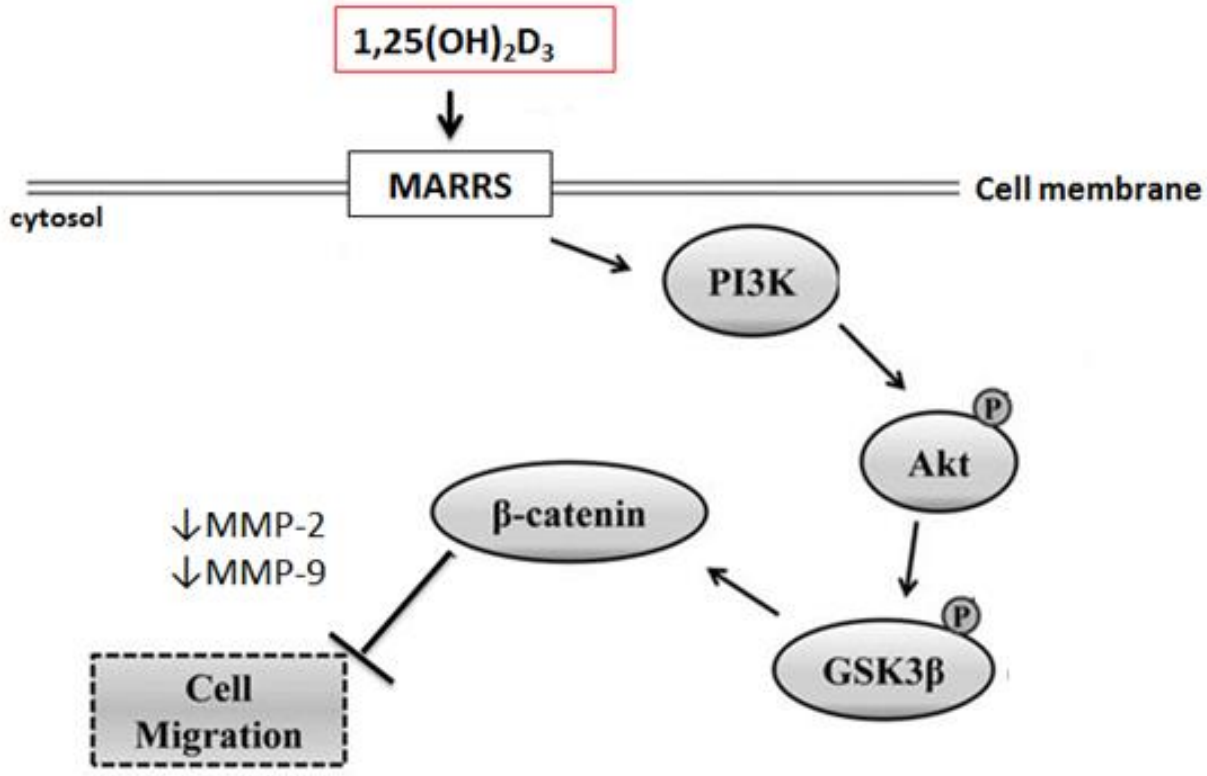


Figure 4-3: PI3K/Akt/GSK3β signalling models to explain the non-genomic response of dermal fibroblasts to $1,25(OH)_2D_3$. $1,25(OH)D_3$ binding to MARRS results in the activation of PI3K. Inactivation of GSK3β results in an accumulation of cytosolic β-catenin, with reduced nuclear β-catenin level. A reduction in nuclear β-catenin expression decreases MMP-2 and MMP-9 secretion, thereby inhibiting dermal fibroblast migration. Adapted from (Li et al. 2017)

Serum contains albumin and growth factor that can mask the effect of $1,25(OH)_2D_3$ on cell migration

To ensure that the cellular responses observed were due to autocrine or paracrine properties of the cells themselves, primary cultures of dermal fibroblasts were cultured in serum-free condition. The use of serum-free media is to avoid binding of $1,25(OH)_2D_3$ and its precursors to plasma proteins such as albumin that could confound evaluation of cell function and response (Stevenson 2005). Dermal fibroblasts were able to maintain normal growth characteristics in serum-free condition for up to 48 hours (Thornton 1990). However, as the fibroblasts used in this study were already serum starved for 24 hours prior to treatment in serum-free media, all the experiments were only carried out for a further 24 hours period (Lu et al. 2017). Beyond 48 hours of serum starvation, the number of surviving cells gradually decreases (Inoue et al. 1999). The scratch wound assays in dermal fibroblasts were repeated in 10% fetal bovine serum as a positive control. In agreement with previous study by Brink *et al* (2005), there is an increase in migration by dermal fibroblast in the presence of serum (Figure 3-4) (Brink et al. 2005). The inhibitory effect of $1,25(OH)_2D_3$ on migration of dermal fibroblasts was masked up to 24 hours as serum contains growth factors that can stimulate cell migration (Stevenson 2005).

Cholecalciferol or $25(OH)D_3$ did not modulate dermal fibroblast migration

Although dermal fibroblast express vitamin D_3 metabolising enzymes at the transcriptional level, incubation with either cholecalciferol or $25(OH)D_3$ did not modulate human dermal fibroblast migration in this study (Figure 3-6). This could be due to the inability of cultured dermal fibroblasts to metabolise both precursors to the active form, $1,25(OH)_2D_3$. There are three possible explanations for this observation. Firstly, keratinocyte-fibroblast paracrine interaction may be essential to initiate vitamin D_3 metabolism *in vivo* (Sato et al. 1997; Witte and Kao 2005). As there are no co-culture studies that specifically examine the paracrine regulation of vitamin D_3 synthesis in the skin, it is difficult

to determine whether this inability could just be due to a lack of paracrine signalling from the neighbouring cells (Bailey 2010). In the present study, human dermal fibroblasts clearly expressed high levels of VDR, while donor-matched epidermal keratinocytes exhibit a higher expression of the vitamin D_3 metabolism enzymes (Figure 3-1). This would suggest that a paracrine regulatory model of $1,25(OH)_2D_3$ synthesis is a highly plausible model. The second explanation is that even though there may be some *de novo* synthesis of $1,25(OH)_2D_3$ from the precursors, the final concentration of the active metabolites from this *de novo* synthesis could be too low or may take longer than 24 hours to exert any biological effect in culture conditions. Thirdly, CYP11A1 metabolising pathway as suggested by Slominski *et al* (2014) could have taken place, resulting in the production of other metabolites such as $20(OH)D_3$ and $20,23(OH)_2D_3$, which only serve as partial agonist of the VDR (Figure 1-11) (Slominski et al. 2014).

The lack of inhibition on dermal fibroblast migration when incubated with the precursors of $1,25(OH)_2D_3$ can also be explained by their lack of binding affinity to the VDR (Lips 2007; Ohyama and Shinki 2016). Although $25(OH)D_3$ is the major circulating form of vitamin D in humans, it has a lower affinity to VDR than $1,25(OH)_2D_3$. In human tissue, the VDR binds $25(OH)D_3$ 100 times less effectively than $1,25(OH)_2D_3$ (Bouillon et al. 1995; Chawla et al. 2001; Lips 2007). The reduced binding affinity can be attributed to the 5.8-16.1% larger VDR-Ligand Binding Pocket (VDR-LBP) of $25(OH)D_3$ compared to that of the $1,25(OH)_2D_3$, thus creating less tension and pressure in the ligand binding pocket (Lou et al. 2010). In addition, $25(OH)D_3$ can form fewer hydrogen bonds with the VDR due to a lack of the 1,hydroxyl-group (Lou et al. 2010). On the other hand, cholecalciferol does not bind to VDR and hence cannot control gene expression via the VDR (Chen et al. 2000; Ohyama and Shinki 2016).

4.3 $1,25(OH)_2D_3$ or its precursors did not modulate metabolic activity of human dermal fibroblasts

An Alamar Blue assay was used to assess the metabolic activity of cultured human dermal fibroblasts. It is a useful assay to determine whether dermal fibroblasts remain viable and retain their proliferative activity *in vitro* (Al-Nasiry et al. 2007). There was no demonstrable difference in the metabolic activity of human dermal fibroblasts after 24 hours in the presence of 10nM of $1,25(OH)_2D_3$ or cholecalciferol or $25(OH)D_3$. In view of this, it is safe to conclude that dermal fibroblasts used in this study were all still viable after incubation in serum free media for 48 hours and the treatment media did not alter cell viability or proliferation significantly for up to 24 hours. However, it is possible that the effect of $1,25(OH)_2D_3$ and its precursors on fibroblast metabolic activity are different over a longer time period, as they enter into different stages of wound healing.

4.4 VDR nuclear expression was upregulated in scratched human dermal fibroblast, in the presence of $1,25(OH)_2D_3$

VDR is a nuclear transcription factor (Pike and Meyer 2010). It is activated mainly through binding with $1,25(OH)_2D_3$ (Lips 2007; Pike and Meyer 2010). Upon binding with $1,25(OH)_2D_3$, VDR forms a heterodimer with RXR which results in the nuclear import of VDR (Yasmin et al. 2005). All genomic actions of $1,25(OH)_2D_3$ are mediated by VDR and these genomic actions give rise to the majority of the effects of this hormone (Carlberg and Campbell 2013). In the present study, incubation of dermal fibroblasts with $1,25(OH)_2D_3$ on their own did not increase VDR nuclear expression (Figure 3-8). However, when coupled with mechanical wounding, an upregulation of VDR nuclear expression was observed, indicating that mechanical wounding of dermal fibroblasts regulates transcriptional activities of ligand activated-VDR. An example of transcriptional regulatory factors that has been shown to be consistently upregulated after injury is activator protein (AP)-1 family of transcription factors (Aslam et al.

1999; Yates and Rayner 2002; Gangnuss et al. 2004; Gangnuss et al. 2004; Neub et al. 2007; Landén et al. 2016). AP-1 is a group of transcription factors that typically binds as to a common enhancer sequence on target gene promoters (McDonald 2004). In fact, interaction between VDR and AP-1 has been well documented (Aslam et al. 1999; Kongsbak et al. 2013). AP-1 mediated trans-activation of VDR is possibly to result in the upregulation of VDR nuclear expression observed in the present study (Qi et al. 2002; Zenz et al. 2008).

Secondly, the increase in nuclear expression of VDR could be explained by an increase in binding of co-activators to VDR-RXR heterodimer. Mechanical wounding results in the release of various cytokines (e.g. TGFβ and IL-6) and growth factors (e.g. FGF, EGF, and VEGF) from dermal fibroblast (Werner and Grose 2003; Stevenson 2005; Barrientos et al. 2008; Behm et al. 2012; Xu et al. 2013). These cytokines and growth factors stimulate diverse signalling cascades within the dermal fibroblast. A common denominator in these signal transduction pathway is the activation of members of the Src-kinase family (Row et al. 2005). The Src co-activator stabilises VDR to RNA polymerase II and the basal transcription machinery binding (Zhang et al. 2001). Binding of Src also leads to recruitment of transcription factors (e.g. TFII/TATA protein) and transcription activators (CBP/p300) which further enhances VDR transcriptional activities (Figure 1-15) (Kamei et al. 1996; Takeshita et al. 1996; Yao et al. 1996).

Thirdly, an increase in cytoplasmic-nuclear cycling of the VDR could partly explain the increase in VDR nuclear expression. At basal level, VDR mainly resides in the cytoplasm (Ruth Wu-Wong et al. 2006). In agreement with our result (Figure 3-8), previous study using enterocyte-like cell line has shown that incubation with 100nM $1,25(OH)_2D_3$ does not result in an increase in nuclear VDR expression (Klopot et al. 2007). However, an increase in nuclear VDR was observed after chemical damage (induced by photobleaching), in the presence of $1,25(OH)_2D_3$ (Klopot et al. 2007). The increase in nuclear translocation of

VDR is possibly to be mediated via recruitment of importin α to the VDR (Prufer and Barsony 2002; Yasmin et al. 2005; Klopot et al. 2007). Upon binding to the nuclear localisation sequence on VDR, importin α directs the mobilization of VDR-RXR heterodimer through the nuclear pore into the nucleus, manifested as an increase in nuclear VDR expression (Figure 3-8) (Morrill et al. 2016)

Cholecalciferol or $25(OH)_2D_3$ did not modulate VDR nuclear expression

In contrast, incubation of human dermal fibroblasts with cholecalciferol or $25(OH)D_3$ did not increase VDR nuclear expression, in both non-scratched and scratched conditions (Figure 3-9 and Figure 3-10). This concurs with the finding in the migration assay, which showed that the precursors did not modulate dermal fibroblast migration, further supporting the hypothesis that dermal fibroblast is not efficient in autocrine production of $1,25(OH)_2D_3$ from their precursors in culture.

4.5 Knockdown of VDR by siRNA abolishes the inhibitory effect of $1,25(OH)_2D_3$ on dermal fibroblast migration in a scratch wound assay

In order to understand the mechanism behind the inhibition of dermal fibroblast migration in response to $1,25(OH)_2D_3$ and the role of the VDR in modulating that effect, the migration study was repeated with the introduction of VDR small interfering RNA (siRNA). Knockdown of VDR was highly efficient with the introduction of VDR siRNA in dermal fibroblast, showing a 89-91% reduction of VDR mRNA expression in comparison to the non-target siRNA control (Figure 3-11). Based on previous studies, a period of at least 24 hours incubation with VDR siRNA is necessary for successful fibroblast uptake of the guide strand of the siRNA into an RNA-induced silencing complex (RISC) that directs degradation of VDR mRNA (Janjetovic et al. 2009; Pratt and MacRae 2009; Holmes et al. 2010). In view of this, all transfection with siRNA was performed for 24 hours prior to commencing the scratch wound assay. In the present study, silencing of VDR abrogates the inhibitory effect of $1,25(OH)_2D_3$

on dermal fibroblast migration (Figure 3-12). In concur with the increase in VDR nuclear expression as shown in the immunofluorescence study (Figure 3-8), this further confirms the hypothesis that the inhibitory effect of $1,25(OH)_2D_3$ on dermal fibroblast migration is primarily a genomic response, mediated via the VDR.

4.6 $1,25(OH)_2D_3$ and its precursors modulate mRNA expression of VDR and CYPs in epidermal keratinocytes and dermal fibroblasts

Scratching did not modulate relative mRNA expression of VDR and CYPs (Figure 3-13)

To elucidate whether the process of wounding modulate the expression of VDR and CYPs in epidermal keratinocytes and dermal fibroblasts, quantitative real-time PCR was performed. Scratching in itself does not modulate VDR or CYP2R1, CYP27B1 and CYP24A1 level of mRNA expression, in both keratinocytes and fibroblasts. Nevertheless, in the presence of exogenous $1,25(OH)_2D_3$ or its precursors, the level of mRNA expression of CYPs changes in both cells, but VDR is only upregulated in dermal fibroblasts and remains unchanged in epidermal keratinocytes (Table 3-1).

$1,25(OH)_2D_3$ and its precursors modulate VDR and CYPs mRNA expression

Previous studies have shown that the CYP24A1 gene is highly inducible by $1,25(OH)_2D_3$ (Lu et al. 2017). In fact, it is the most strongly regulated VDR-target gene, with its promoter containing multiple VDREs (Väisänen et al. 2005; Gocek et al. 2014). Meyer *et al* (2017) showed that $1,25(OH)_2D_3$ induces rapid VDR-RXR binding to the VDREs containing the CYP24A1 gene, and the upregulation is dose-dependent (Meyer et al. 2007). In the present study, incubation with either 10nM of $1,25(OH)_2D_3$ or cholecalciferol, or $25(OH)D_3$, all resulted in an upregulation of CYP24A1 expression in the dermal fibroblast. The same was also observed in the keratinocytes when they were incubated with

10nM $1,25(OH)_2D_3$, which is in agreement with a study by Schuessler *et al* (Schuessler et al. 2001). CYP24A1 is a mitochondrial inner-membrane cytochrome P450 enzyme that exhibits multiple catalytic functions (St-Arnaud 2011). It plays a major role in the catabolism of $25(OH)D_3$ or $1,25(OH)_2D_3$ to their 'inactive' form, to prevent tissue-level $1,25(OH)_2D3$ intoxication (Hosogane et al. 2003; Jones 2008). This is achieved via hydroxylation of the C23 or the C24 side-chain carbons of $25(OH)D_3$ or $1,25(OH)_2D_3$ (St-Arnaud 2011). In humans, the 24-hydroxylase activity predominates with $24,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$ being the major products (Bikle 2017). In the present study, by upregulating CYP24A1 (Table 3-1), rapid degradation of $25(OH)D_3$ and $1,25(OH)_2D_3$ helps to reduce the ability of these two metabolites to continuously activate VDR-dependent and VDR-independent pathways that could be detrimental to cell survival and proliferation (Pols et al. 1990; Girgis et al. 2014). This CYP24A1 upregulation effect in response to incubation with $1,25(OH)_2D_3$ is conserved over a wide variety of human cell types (e.g. epidermal keratinocytes, dermal fibroblasts, osteoblasts, colonic cells), signifying the importance of fine tuning of local bioavailability of $1,25(OH)_2D_3$ in the dermis (Zierold et al. 1995; Schuster et al. 2001; Díaz et al. 2015; Norlin et al. 2017).

The present study has shown that 10nM of cholecalciferol increases VDR mRNA expression in scratched dermal fibroblasts (Table 3-1). This finding concurs with the result of a study by Tokar *et al* (2005) which demonstrated that cholecalciferol upregulates VDR expression (Tokar and Webber 2005). However, it is important to note that in the current study, an upregulation of cholecalciferol mRNA transcription did not translate into an increase in nuclear VDR protein expression, as demonstrated in the immunofluorescence staining (Figure 3-9). A functional study using the scratch wound assay also showed a lack of phenotypic changes to dermal fibroblast migration following incubation with cholecalciferol. This could be attributed to post-transcriptional regulation of VDR protein expression by dermal fibroblast (Greenbaum et al. 2003; Maier et al. 2009; Liu et al. 2016). Further studies such as Western Blotting could

determine the correlation between the cellular mRNA expression and protein expression in dermal fibroblast.

In non-scratched dermal fibroblasts, 10nM cholecalciferol only upregulated CYP27B1 mRNA expression (Table 3-1). This is in contrast with an upregulation of CYP2R1, CYP27B1 and CYP24A1 mRNA expression in scratched fibroblasts. The recently discovered non-classical cholecalciferol metabolising pathway can partly explain these findings (Slominski et al. 2014). CYP11A1 can also catalyse hydroxylation of the side chain of cholecalciferol at C20 with 20(OH)D_3 being the major metabolite (Figure 4-4) (Guryev et al. 2003; Slominski et al. 2005; Tuckey et al. 2008; Tuckey et al. 2011). As CYP11A1 is unable to hydroxylate 20(OH)D_3 further, CYP27B1 is required for further hydroxylation of 20(OH)D_3 to 1,20(OH)$_2D_3$, a compound with active biological functions in epidermal keratinocytes that include reducing proliferation and increasing differentiation . Furthermore, other products downstream of 20(OH)D_3 metabolism also require CYP27B1 for further hydroxylation (Figure 4-4). This could explain the upregulation of CYP27B1 in non-scratched dermal fibroblasts (Table 3-1). The finding that CYP24A1 is not upregulated could be due to the fact that most of the end products of this metabolising pathway such as 20(OH)D_3 and 20,23(OH)$_2D_3$ can only act as partial agonists of the VDR (Slominski et al. 2014). As a result, induction of CYP24A1 expression was not significant as CYP24A1 is a VDR-targeted downstream gene (Väisänen et al. 2005; Gocek et al. 2014). The question still remains as to why the dermal fibroblast utilises the CYP11A1 pathway rather than the classical CYP2R1 pathway in culture. Further studies comparing the relative mRNA expression of CYP11A1 and CYP2R1 by dermal fibroblast could help in our understanding on the preferred cholecalciferol metabolism pathway in human dermis.

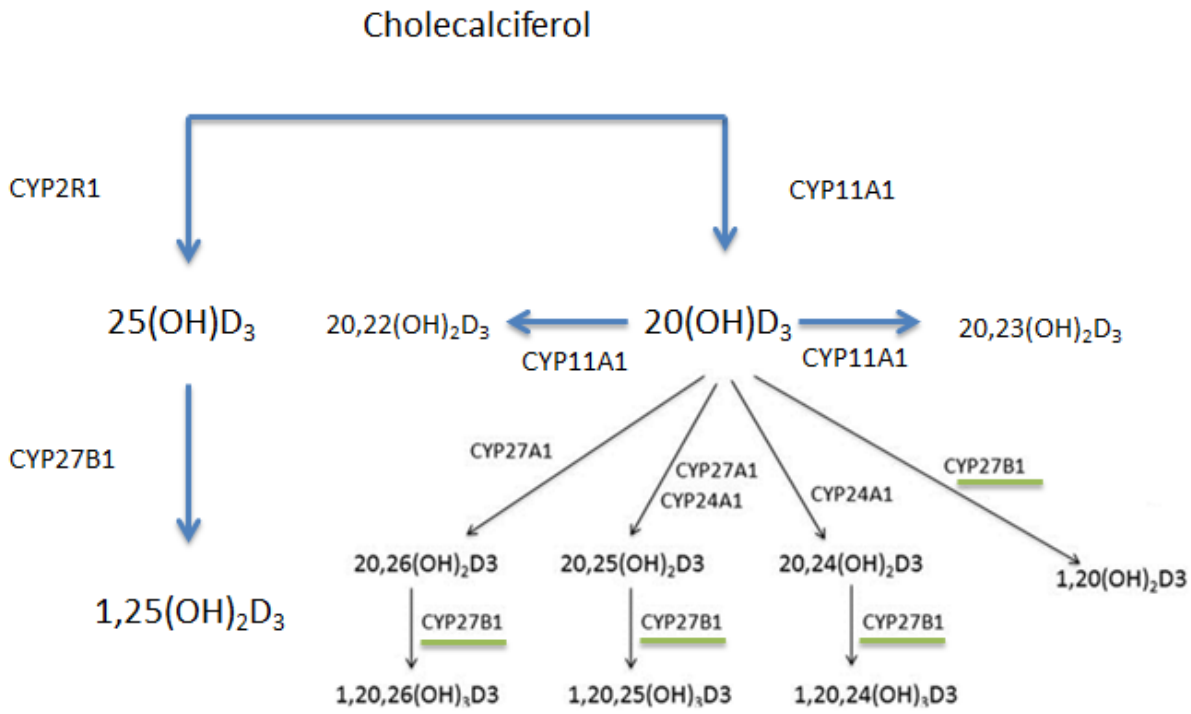


Figure 4-4: The novel, alternative pathway of cholecalciferol metabolism utilising CYP11A1 compared to the classical pathway which involves CYP2R1. The expression of CYP11A1 in non-classical steroidogenic tissues such as skin is relatively low and unlikely to have major systemic effects. Adapted from (Slominski et al. 2014)

4.7 Knockdown of VDR upregulates CYP27B1 in scratched dermal fibroblasts, in the absence or presence of $1,25(OH)_2D_3$

We report, for the first time, the possible existence of a feedback control on the key vitamin D enzymes CYP27B1 through VDR-independent signalling in the human dermal fibroblasts. This is in contrast with previous study using human corneal epithelial cell where VDR silencing and incubation with $1,25(OH)_2D_3$ did not modulate CYP27B1 protein expression (Lu et al. 2017).

Intracellular calcium regulates cellular mechanics in motile fibroblasts following wounding (Evans and Falke 2007; Wei et al. 2009). Acting via myosin

II and calmodulin, calcium has a multifunctional role in traction force generation, directional sensing and relocation of focal adhesions in migrating fibroblasts (Wei et al. 2008). $1,25(OH)_2D_3$ has been shown to down-regulate the L-type voltage-sensitive calcium channels (LVSCC) (Brewer et al. 2001), a possible pathway for non-genomic signalling of $1,25(OH)_2D_3$. Conversely, an upregulation of LVSCC has been observed following VDR knockdown in neuron cells (Gezen-Ak et al. 2011).

Migration assay in the present study showed that incubation with $1,25(OH)_2D_3$ downregulated dermal fibroblast migration as early as 4 hours (Figure 3-5), a non-genomic effect that could be explained via modulation of calcium channel (inhibition of LVSCC expression). Upon VDR silencing, CYP27B1 expression is upregulated by scratched dermal fibroblast in order to produce more $1,25(OH)_2D_3$, with the aim to achieve downregulation of LVSCC as an alternative route to achieve inhibition of fibroblast migration, independent of the VDR.

4.8 10nM $1,25(OH)_2D_3$ downregulates α-SMA in scratched human dermal fibroblasts

Alpha-SMA is a biomarker for differentiation of dermal fibroblasts into myofibroblasts, which are important effector cells of tissue fibrogenesis (Zhao et al. 2018). In a cutaneous wound, myofibroblasts play a central role in wound contraction (Gabbiani 2003; Chitturi et al. 2015). Mechanical stress and TGF-β signalling are two major factors contributing to the transition from fibroblast to myofibroblast (Gabbiani 2003). In this study, immunofluorescence staining for α-SMA was performed to confirm if human dermal fibroblasts increase their expression of this protein in response to mechanical wounding, as previously described (Stevenson 2005; Pomari et al. 2014), and to determine whether $1,25(OH)_2D_3$ or its precursors could modulate it. There was a demonstrable increase in α-SMA staining following scratching at 24 hours, in agreement with Stevenson *et al* (2005) and Pomari *et al* (2014). However, α-SMA protein

expression in scratched dermal fibroblasts was markedly reduced in the presence of 10nM $1,25(OH)_2D_3$ for 24 hours (Figure 3-15 and Figure 3-16). In contrast, there was no demonstrable change in α-SMA expression in response to 10nM of cholecalciferol or $25(OH)D_3$ (Figure 3-15 and Figure 3-16). This is the first study to examine the role of $1,25(OH)_2D_3$ and its precursors on human dermal fibroblast differentiation *in vitro*. It suggests that $1,25(OH)_2D_3$ can modulate the fibroblast phenotype in wounded conditions, in agreement with findings from previous studies using fibroblasts from other organs such as human lung fibroblasts and human renal interstitial fibroblasts (Li et al. 2005; Tan et al. 2007; Zhang et al. 2015). This response may be due a reduction in stress fibre formation, characteristic of myofibroblast activation (Gabbiani 2003; Meredith et al. 2015). In addition, $1,25(OH)_2D_3$ can down-regulate transforming growth factor (TGF)-β and indirectly modulating its downstream effectors SMAD2 and SMAD3 which regulate the target gene (α-SMA) involved in myofibroblast differentiation (Shin et al.; Ding et al. 2013). Once again, cholecalciferol and $25(OH)D_3$, both precursors of $1,25(OH)_2D_3$ failed to modulate α-SMA expression *in vitro*, supporting the hypothesis that cultured dermal fibroblasts are ineffective in autocrine production of active $1,25(OH)_2D_3$.

4.9 $1,25(OH)_2D_3$ downregulates soluble MMP-2 activity while cholecalciferol has the opposing effects, in scratched dermal fibroblasts

MMPs are a group of proteases that play important roles during the wound healing process (Xue et al. 2006). MMPs regulate inflammatory processes and degrade the extracellular matrix to facilitate remodelling of the ECM and cutaneous wound closure (Caley et al. 2015; Rohani and Parks 2015). Specifically, an interaction between vitamin D_3 and MMP-2/MMP-9 in fibroblasts (derived from nasal polyps) has been demonstrated (Wang et al. 2015). Using ELISA and Western blot analysis, Wang *et al* (2015) showed that TNF-α-induced MMP-2 and MMP-9 productions are inhibited by $1,25(OH)_2D_3$ after 24 hours. MMP-2 and MMP-9 are both gelatinases, therefore gelatine zymography

was utilised to assess the activity of soluble MMP-2 and MMP-9 secreted by human dermal fibroblasts at basal conditions and upon scratching (Figure 3-17). This present study also investigated the relative activity of both MMPs in response to $1,25(OH)_2D_3$ and cholecalciferol (Figure 3-17).

MMP-9 activity remained undetectable in gelatine zymography (Figure 3-17), which could be due to the relatively low concentration of MMP-9 in the conditioned media collected from the cells cultured in 6-well plates. Similar experiment could be performed on dermal fibroblasts cultured in larger flasks such as in T75 or T225 flasks, to obtain a higher amount of secreted MMP-9. An alternative would be to use other sensitive techniques such as RT-PCR or Western Blot to assess MMP-9 mRNA or protein expression. Similar finding was reported in a study by Singer *et al* (2002) using breast cancer-derived human fibroblasts. They suggested that low MMP-9 activity is attributed to a lack of stromal-epithelial cell interaction *in vitro* (Singer et al. 2002). Co-culture of epidermal keratinocytes and dermal fibroblasts or using reconstructed human skin model would be useful methods to elucidate whether mesenchymal-interaction is necessary to stimulate MMP-9 production by fibroblasts.

In scratched dermal fibroblasts, incubation with 10nM of cholecalciferol significantly upregulated MMP-2 whereas 10nM of $1,25(OH)_2D_3$ downregulated it (Figure 3-17). This appears to be a paradoxical finding, but the alternative CYP11A1-mediated cholecalciferol metabolism pathway could partly explain it (Figure 4-4). Cholecalciferol is possibly to be metabolised to other CYP11A1-derivatives which exhibit different effects as compared to that shown by $1,25(OH)_2D_3$. Table 4-1 summarises the phenotypic effects of some of the CYP11A1-derived hydroxyderivatives of vitamin D_3 on epidermal keratinocytes and dermal fibroblasts *in vitro* (Slominski et al. 2014). As demonstrated by the work of Slominski *et al* (2014), derivatives from CYP11A1-mediated metabolism of cholecalciferol downregulates TGF-β expression (Slominski et al. 2014). As TGF-β is known to suppress the expression of MMP-2, an inhibition of TGF-β by these derivatives result in higher MMP-2 activity (Risinger et al. 2010).

On the other hand, 10nM of 1,25$(OH)_2D_3$ downregulated MMP-2 activity in scratched dermal fibroblasts (Figure 3-17). As the MMP-2 downregulatory effect was only observed in scratched dermal fibroblasts, but not in non-scratched fibroblasts (Figure 3-17), it could be postulated that an overall decrease in MMP-2 secretion is beneficial for fibroblasts under wounded conditions. A decrease in MMP-2 secretion could be important to prevent excessive breakdown of extracellular matrix at wound site commonly associated with inflammation, thereby helping to accelerate wound closure. This finding (Figure 3-17) also provides supportive evidence to the hypothesis as discussed in section 4.2 where 1,25$(OH)_2D_3$ could mediate a decrease in MMP-2 protein expression in dermal fibroblasts that manifested as an inhibition in fibroblast migration (Figure 4-2).

Vitamin D_3 derivatives from CYP11A1 metabolism	Human dermal fibroblasts		Human epidermal keratinocytes	
	*Fibrosing effect	Proliferation	Differentiation	Proliferation
20(OH)D_3	↓	↓	↓	↓
20,23(OH)D_3	↓	↓	↓	↓
22(OH)D_3 and 20,22(OH)$_2D_3$	ND	↓	↑	↓
17,20,23(OH)$_3D_3$	ND	↓	ND	↓
20,24(OH)$_2D_3$, 20,25(OH)$_2D_3$ and 20,26(OH)$_2D_3$	ND	ND	ND	ND
1,20(OH)$_2D_3$	ND	↓	↑	↓
1,20,25(OH)$_3D_3$ and 1,20,26(OH)$_3D_3$	ND	ND	ND	ND

Table 4-1: All primary cell used in the experiments were derived from female (F) facial skin. ↑ **-** stimulation; ↓ - inhibition, * - defined as inhibition of TGF-β induced hyaluronate and collagen production. All primary cells were derived from scleroderma and normal donors, ND- not determined. Adapted from (Slominski et al. 2014).

4.10 1,25(OH)$_2$D$_3$ downregulates the type I to III collagen ratio in scratched dermal fibroblasts.

As fibroblasts differentiate into myofibroblasts, they secrete collagen I and III, two of the major components of ECM (Klingberg et al. 2013). To investigate the effect of 1,25(OH)$_2$D$_3$ on collagen secretion by dermal fibroblasts and how this may be regulated in the context of wound healing, intact and mechanically wounded dermal fibroblasts were cultured in the presence or absence of 1,25(OH)$_2$D$_3$. The relative mRNA expression of collagen I and collagen III was determined using qRT-PCR.

Depending on the depth of the dermal layer, papillary and reticular dermal fibroblasts exhibit differences in their gene expression (Sorrell and Caplan 2004; Janson et al. 2012). In the present study, only the papillary dermal fibroblasts were used as they are closer to epidermal keratinocytes anatomically and thus more possibly to be involved in epithelial-mesenchymal paracrine regulation of vitamin D$_3$ homeostasis. Mechanical scratching of cultured human dermal fibroblasts upregulated collagen type I mRNA expression after 24 hours, but had no effect on type III collagen mRNA expression (Figure 3-18). This finding concurs with the observation by Meigel *et al* (1997) where type I collagen is the predominant collagen type in the papillary dermis, whereas type III collagen constitutes a large proportion of the reticular dermis (Meigel et al. 1977; Gay et al. 1980; Haukipuro et al. 1991; Ali Bahar et al. 2004). Although there are some studies which reported that type III collagen expression is higher during the early stages of wound healing, these studies were done on murine fibroblasts, and did not specifiy the type of dermal fibroblast, i.e. papillary or reticular, that were used (R Merkel et al. 1988; Volk et al. 2011).

1,25(OH)$_2$D$_3$ significantly upregulated type I collagen mRNA expression in non-scratched dermal fibroblasts at both 10nM and 100nM, thereby increasing the collagen type I to type III ratio (Figure 3-19). This finding is in agreement with the results shown by Dobak *et al* (1994) where 1,25(OH)$_2$D$_3$ significantly increased collagen type I and type III production by neonatal foreskin fibroblast as measured by ELISA (Dobak et al. 1994). In normal skin, decreasing levels of

type I and III collagen production, and the type I to III collagen ratio are associated with ageing, and associated loss of tension and elasticity in the skin (Cheng et al. 2011). This indicates the potential use of $1,25(OH)_2D_3$ to counter the effects of collagen loss associated with ageing. In fact, murine studies have shown that VDR-knockout mice developed premature skin ageing phenotype with thinning of skin and wrinkling (Keisala et al. 2009; Tuohimaa 2009).

In scratched dermal fibroblasts, $1,25(OH)_2D_3$ downregulated both collagen type I and type III mRNA expression, which was also accompanied by a decrease in the type I to III ratio (Figure 3-19). A higher type I to III collagen ratio is associated with abnormal hypertrophic scarring, and studies have also shown that vitamin D deficiency stimulates fibroblasts transition into myofibroblasts in Dupuytrens's disease (profibrotic changes in skin) (Cheng et al. 2011; Shi et al. 2013; Seyhan et al. 2018). Taken together with the effect of $1,25(OH)_2D_3$ in abrogating myofibroblast differentiation as shown in the immunofluorescence study (Figure 3-15), this indicates that $1,25(OH)_2D_3$ may be able to modulate the scarring process and promote wound remodelling (Lee et al. 2017).

4.11 Incubation with $1,25(OH)_2D_3$ increases the rate of wound closure in human skin *ex vivo*

The beneficial role of vitamin D_3 in cutaneous wound healing has been shown in human clinical trials where reposition of vitamin D in patients with vitamin D insufficiency speeds up the healing of venous ulcers (Burkiewicz et al. 2012). However, no work has been done to delineate the exact molecular mechanisms by which $1,25(OH)_2D_3$ exerts its effects on different components of human skin using a human *ex vivo* wound healing model. The model used in the current study was adapted from Stojadinovic and Tomic-Canic (2013).

Human full-skin *ex vivo* models have been used extensively in the past for studies investigating the expression of cytokines in psoriatic skin, the behaviour of epidermal Langerhan cells and functional studies of the role of

epidermal growth factor (EGF) in psoriasis (Rambukkana et al. 1995; Yoshinaga et al. 1995; Varani et al. 1998). An *ex vivo* model mimics the three dimensional wound environment and can be utilised to investigate the paracrine regulation of vitamin D at the wound site and its relationship to the rate of wound healing. The main limitation of any *ex vivo* wound healing model is that it does not have an endogenous blood supply. Nutritional supply to the dermal fibroblasts and epidermal keratinocytes depends on passive diffusion of nutrients from the media into the cells. It has been shown previously that incubation with 10% FBS is sufficient for complete re-epithelialisation of human *ex vivo* cutaneous wounds in culture (Kratz 1998). Hence, in this study 10% FBS was used as a positive control to study the stimulatory effects on the rate of wound closure in the presence $1,25(OH)_2D_3$ and cholecalciferol (Figure 3-21, Figure 3-22 and Figure 3-23). *De novo* re-epithelialisation was confirmed using H&E staining on cryosections of the wounded skin explants (Figure 3-20). The increase in granulation tissues density at wound bed over the 6 days period (Figure 3-20) also supports the use of this *ex vivo* wound healing model as a feasible model to study the wound healing response.

The present study used whole skin cultures derived from two different anatomical sites (abdominal and breast), from female donors of different ages (34 years and 59 years). The results showed that $1,25(OH)_2D_3$, either in the presence or absence of serum, accelerated wound closure *ex vivo* after three to four days (Figure 3-21, Figure 3-22 and Figure 3-23). In the *in vitro* study, it was shown that $1,25(OH)_2D_3$ increased epidermal keratinocyte migration (Figure 3-3), but in contrast inhibited dermal fibroblast migration (Figure 3-4 and Figure 3-5). While these results suggest that dermal fibroblast migration may not be an important determinant in the up-regulation of wound closure, it must be considered that fibroblasts may respond differently in a 2-dimensional monolayer environment than they would in a three-dimensional wound setting. Recent studies have shown that dermal fibroblasts behave differently when allowed to form 3-dimensional cultures e.g. cultured on scaffolds when compared to their 2-dimensional counterparts cultured in monolayers (Rothan et

al. 2014; Smithmyer et al. 2014). It would be interesting to carry out further studies to compare the response of dermal fibroblasts to 1,25$(OH)_2D_3$ in 2-D and 3-D culture. For example, wounded dermal fibroblast differentiation into myofibroblast is potentially to be upregulated at basal condition in a 3-D culture, owing to the external stress/tension generated by the three-dimensional collagen lattices (Kessler et al. 2001; Rothan et al. 2014). In addition, secretion and deposition of collagen fibres is potentially to be upregulated due to cell-matrix interactions and an increase in cell-cell interaction across three dimensions (Maione et al. 2015).

Other factors such as, epithelial-mesenchymal interactions, the inflammatory response, granulation tissue formation and modulation regulated by dermal fibroblasts may be more essential for wound closure and promoting re-epithelialisation (Garner 1998; Wang et al. 2015). Co-cultures of epidermal keratinocytes and dermal fibroblasts using a Transwell® inserts system could help to investigate the paracrine regulation of key enzymes, cytokines and growth factors involved in wound closure, in response to 1,25$(OH)_2D_3$ (Wang et al. 2012). On the other hand, it has been well established that 1,25$(OH)_2D_3$ is a potent inducer of cathelicidin, an antimicrobial peptide that helps to fight infection, which is important during early phases of wound healing (Gombart 2009). Furthermore, 1,25$(OH)_2D_3$ has been shown to reduce inflammation via regulation of the inflammatory cytokines (e.g. TNF-α and IL-1) and inhibiting the proliferation of proinflammatory cells such as macrophages and neutrophils (Takahashi et al. 2002; Du et al. 2009; Kuo et al. 2010; Yin and Agrawal 2014). Further studies investigating the level of inflammatory cytokines secreted by scratched dermal fibroblasts in response to 1,25$(OH)_2D_3$ would be useful to delineate the molecular mechanism by which vitamin D_3 helps in resolving excessive inflammation in cutaneous wound, a process if unchecked, could lead to chronic wounds and abnormal scarring.

In contrast, cholecalciferol did not alter the rate of wound closure (Figure 3-21 and Figure 3-22). This suggests that epidermal keratinocytes and dermal

fibroblasts lack the ability to metabolise exogenous cholecalciferol into $1,25(OH)_2D_3$ in an *ex vivo* wound healing model. If the CYP11A1 pathway has been preferentially activated to metabolise cholecalciferol, this also suggests that derivatives from CYP11A1-mediated metabolism are not effective in stimulating wound closure in an *ex vivo* model. In the present study, an upregulation of CYP2R1 and CYP27B1 were not observed following scratching of epidermal keratinocytes *in vitro* (Figure 3-13). This indicates that wounded epidermal keratinocytes does not upregulate the metabolism of cholecalciferol or $25(OH)_2D_3$ into $1,25(OH)_2D_3$ in culture. This could be due to prioritisation of metabolic energy expenditure by wounded epidermal keratinocytes to activities such as cell migration and proliferation with the aim of re-establishing epithelial barrier.

Approximately 99% of circulating $25(OH)D_3$ and $1,25(OH)_2D_3$ are bound to the vitamin D binding protein (DBP) or albumin under normal physiological conditions (Yousefzadeh et al. 2014). In humans, both passive diffusion across cell membrane and active endocytosis via cell surface receptors help in the uptake of $25(OH)D_3$ and $1,25(OH)_2D_3$ into cells (Chen and Farese 1999; Nykjaer et al. 1999). Hence, in the presence of 10% FBS, some of the $1,25(OH)_2D_3$ may be bound, which could affect its bioavailability and therefore its ability to elicit a response in the *ex vivo* model. This could partly explain the lack of synergistic effect in further accelerating *ex vivo* wound closure rate at day 3 when incubated with both FBS and $1,25(OH)_2D_3$ (Figure 3-21).

Cytokines and proteolytic enzymes secreted locally at the wound site in response to wounding could have potentially denatured some of the exogenous cholecalciferol and $1,25(OH)_2D_3$ before they reach the cells to exert any effects. This may be a limitation of this model. Lastly, prolonged elevated expression of MMP can lead to excessive ECM degradation and impair wound closure. As shown in our gelatine zymography study, $1,25(OH)_2D_3$ is able to downregulate MMP-2 activity by scratched dermal fibroblast (Figure 3-17). This is evidently important to allow rapid formation of granulation tissue that acts as tissue

scaffold to support early re-epithelialisation process, as demonstrated in our H&E staining (Figure 3-20).

5 CONCLUSIONS

For decades, vitamin D has been employed as a therapeutic agent for skin disorders such as psoriasis (MacLaughlin et al. 1985; Trémezaygues and Reichrath 2011). Its immunomodulatory roles related to the production of anti-microbial peptides such as cathelicidin and defensin are now well established (Wang et al. 2004). It has also been suggested that cathelicidin can act as an enhancer of the epithelial barrier, and thereby could potentially aid in re-epithelialisation of skin (Niyonsaba et al. 2006; Otte et al. 2009; Schwalfenberg 2011). Despite the apparent importance of skin as an important source and target organ for vitamin D, studies specifically looking at the roles of vitamin D in cutaneous wound healing remain sparse.

The human cutaneous epidermis is a unique site of vitamin D_3 production as keratinocytes contain all the machinery necessary for the autocrine production of bioactive vitamin D (Schuessler et al. 2001). Expression of vitamin D receptors in both epidermal keratinocytes and dermal fibroblasts shows that they are also important target cells. Using both donor-matched and non donor-matched epidermal keratinocytes and dermal fibroblasts, this study has shown for the first time that dermal fibroblasts have a higher relative mRNA expression of VDR, suggesting they are more responsive than epidermal keratinocytes to $1,25(OH)_2D_3$. . In contrast, epidermal keratinocytes express higher levels of mRNA for the CYPs required for the metabolism of vitamin D_3. This high expression in keratinocytes indicates a paracrine, rather than an endocrine source of $1,25(OH)_2D_3$ could be more important for regulating vitamin D_3 bioavailability in dermal fibroblasts.

While $1,25(OH)_2D_3$ stimulated human epidermal keratinocyte migration at 24 hours after mechanical scratching in contrast, it inhibited the migration of human dermal fibroblasts, as early as 4 hours and for up to 24 hours after scratching. The difference in the response time to $1,25(OH)_2D_3$ could be explained by both genomic and non-genomic actions of $1,25(OH)_2D_3$. The VDR

plays a central role in mediating genomic responses. This is clearly demonstrated by the immunofluorescent staining of VDR in dermal fibroblasts incubated with 1,25$(OH)_2D_3$ which markedly increased nuclear VDR protein expression without a corresponding increase in VDR mRNA transcription. The nucleo-cytoplasmic cycling of VDR following ligand activation by 1,25$(OH)_2D_3$ and subsequent association with retinoid X receptor allows the VDR-RXR complexes to interact with vitamin D response elements and regulate target genes transcription (Klopot et al. 2007). The stimulation of epidermal keratinocyte migration was only observed after 24 hours, indicating a genomic response. Furthermore, VDR knockdown by siRNA abolished the inhibitory effect of 1,25$(OH)_2D_3$ on dermal fibroblast migration, suggesting that the anti-migratory effect in dermal fibroblasts was mediated largely via the genomic pathway, with a non-genomic pathway possibly only playing a small part.

Findings from the Alamar Blue assay confirmed that the inhibition of migration observed in dermal fibroblasts was not due to cell death or a reduction in dermal fibroblast proliferation capability following incubation with 1,25$(OH)_2D_3$ or its precursors. Mechanical scratching of monolayers of dermal fibroblasts, in the absence of 1,25$(OH)_2D_3$ did not modulate the mRNA expression of VDR or the CYP enzymes involved in vitamin D_3 metabolism. This suggests that the active ligand, 1,25$(OH)_2D_3$, is needed to stimulate a response by the dermal fibroblast during wound healing. This hypothesis is supported by the immunofluorescence finding that an increase in nuclear translocation of VDR was only observed in scratched dermal fibroblasts, and only in the presence of 1,25$(OH)_2D_3$.

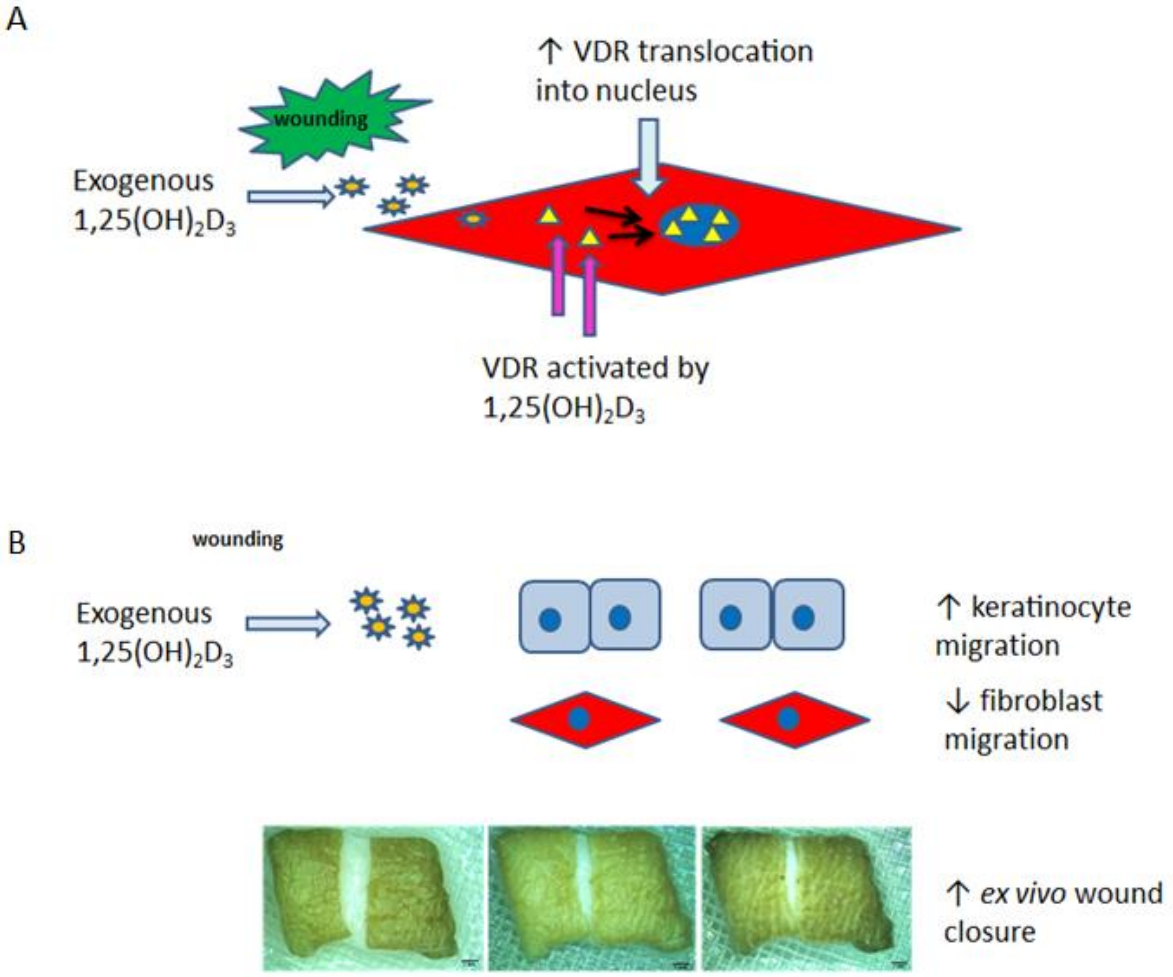
Incubation with 1,25$(OH)_2D_3$ and its precursors significantly up-regulated CYP24A1 mRNA expression in both epidermal keratinocytes and dermal fibroblasts, suggesting that a finely-tuned regulatory system is in place to convert excess 1,25$(OH)_2D_3$ to the inactive form. Even though cholecalciferol and 25$(OH)_2D_3$ down-regulated CYP24A1 expression, their effect was not as pronounced as the modulation observed in the presence of the active form,

$1,25(OH)_2D_3$. This suggests that they or their metabolites have a lower binding affinity to the VDR. This is also supported by the lack of any change in VDR protein expression when dermal fibroblasts were incubated with cholecalciferol or $25(OH)D_3$, or the lack of functional modulation as seen in the migration assay or the *ex vivo* wound healing assay in the presence of cholecalciferol or $25(OH)D_3$ Even though cholecalciferol up-regulated both VDR and CYP enzyme mRNA expression, the effect is possibly to be mediated by the novel CYP11A1 metabolism pathway, where the metabolites such as $20(OH)D_3$ can act as a partial agonist to VDR, in contrast to $1,25(OH)_2D_3$ which has a high binding affinity. This also explains the differences seen with $1,25(OH)_2D_3$ in the modulation of MMP-2 activity, and the migration and *ex vivo* wound healing assays.

Incubation with 10nM $1,25(OH)_2D_3$ decreased α-SMA protein expression in scratched dermal fibroblasts. It also down-regulated MMP-2 activity and the type I to III collagen ratio. Taken together, this suggests that $1,25(OH)_2D_3$ can modulate the phenotypic transition of the fibroblast into a myofibroblast, decrease extra-cellular matrix breakdown by MMP-2 and regulate collagen deposition in the newly formed granulation tissue. Hence, $1,25(OH)_2D_3$ could have a central role in the modulation of wound healing and the scarring process. The findings from the *ex vivo* wound healing model further confirmed that $1,25(OH)_2D_3$ aids in wound closure by enhancing the re-epithelialisation process and deposition of granulation tissue, independently of other growth factors which are present in serum.

In summary, dermal fibroblasts are important target cells for $1,25(OH)_2D_3$. They are more responsive to $1,25(OH)_2D_3$ than epidermal keratinocytes since they express higher levels of VDR transcripts. Even though $1,25(OH)_2D_3$ inhibits dermal fibroblast migration in a monolayer scratch assay, it may have a more important role in extracellular matrix and collagen deposition during the early stages of wound healing. Close epithelial-mesenchymal interaction, in particular, between keratinocytes and fibroblasts, is vital for local

homeostasis of vitamin D_3. The major findings of the study are summarised in Figure 5-1. Further elucidation of the different vitamin D pathways in the skin may lead to improved therapies for wound healing and prevent abnormal scarring.



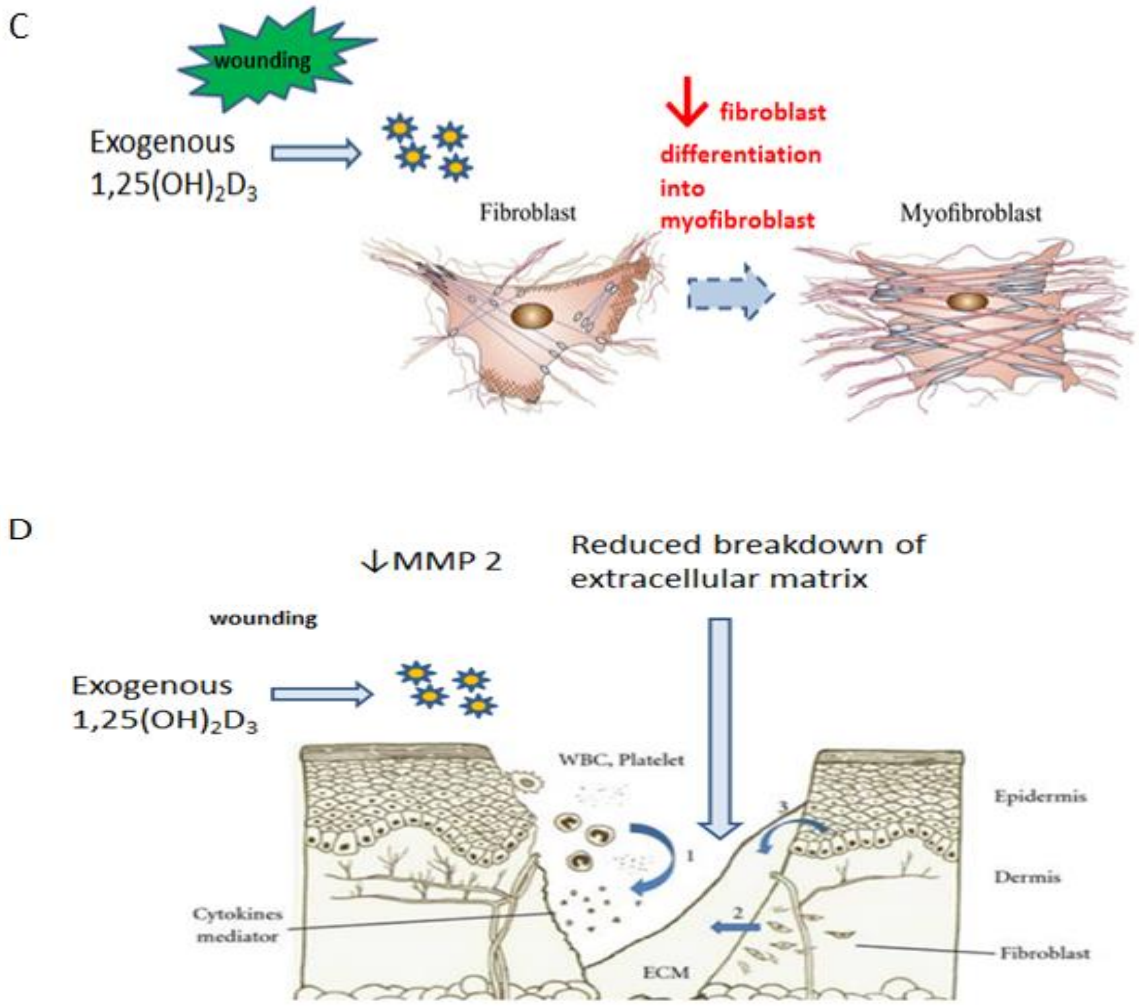


Figure 5-1: Summary of the roles of $1,25(OH)_2D_3$ in cutaneous wound healing. A) $1,25(OH)_2D_3$ upregulated nuclear VDR expression B) $1,25(OH)_2D_3$ increased keratinocyte migration but inhibited fibroblast migration; $1,25(OH)_2D_3$ increased *ex vivo* wound closure C) $1,25(OH)_2D_3$ downregulated α-SMA expression D) $1,25(OH)_2D_3$ downregulated soluble MMP-2 activity. Adapted from (Su et al. 2010; Falke et al. 2015)

6 FURTHER STUDIES

This study has provided strong evidence that both epidermal keratinocytes and dermal fibroblasts express the vitamin D_3 metabolising enzymes at the transcriptional level. The present study also shows that exogenous 1,25$(OH)_2D_3$ is a viable therapeutic agent with the potential to improve cutaneous wound healing with a possible role in modulating the scarring process. However, it also raises questions on the preferred vitamin D_3 metabolic pathways in human skin. In particular, what is the role of CYP11A1 in the production of vitamin D metabolites in the skin? To determine whether the CYP11A1 or the classical CYP2R1 pathway is preferentially activated in epidermal keratinocytes and dermal fibroblasts, qRT-PCR followed by Western Blotting could be performed to compare the relative expression of each cytochrome enzyme at both the mRNA and protein level, following incubation with cholecalciferol. This can be followed by experiments to investigate the synthesis of CYP11A1-derived metabolites by epidermal keratinocytes and dermal fibroblasts *in vitro*, under both wounded and non-wounded conditions. This could be most accurately achieved by performing liquid chromatography and mass spectrometry (LC-MS) analysis to separate and determine the concentrations of expected CYP11A1-derived metabolites, following incubation with cholecalciferol (Slominski et al. 2017).

In addition, it would be interesting to determine the biological effects of the metabolites derived from the CYP11A1 metabolic pathway in the context of cutaneous wound healing. To achieve this, several assays could be performed including the migration assay, collagen gel assay and *ex vivo* wound healing assay. Specifically, 20$(OH)D_3$ and 20,23$(OH)_2D_3$ are the candidate metabolites that are of primary interest due to their partial agonist activity on the VDR. Unfortunately, these metabolites are not commercially available as yet, but they could be synthesised and purified using methods as described by Slominski *et al* (2005) (Slominski et al. 2005).

Thirdly, the interplay between the RXR, VDR and RAR signalling pathways is possibly to be an important regulator in 1,25$(OH)_2D_3$-induced phenotypic changes, as suggested by the migration assay results (Figure 3-3, Figure 3-4, and Figure 3-5). Incubation of epidermal keratinocytes and dermal fibroblasts with 1,25$(OH)_2D_3$ leads to opposing effects in dermal fibroblast migration (Figure 3-3, Figure 3-4 and Figure 3-5). 9-cis retinoic acid, the natural ligand for RXR, is required to stimulate the release of the nuclear co-repressor from VDR-RXR heterodimers (Kang et al. 1997; Sanchez-Martinez et al. 2008). However, as 9-cis RA can bind to both RAR and RXR, the level of RAR expression in keratinocytes and fibroblasts could indirectly determine VDR-dependent gene transcription due to exhaustion of endogenous 9-cis RA (Huang et al. 2013). Loss-of-function studies using gene knockouts of RAR in cultured dermal fibroblasts could provide valuable insight into whether the inhibitory effect on migration seen in dermal fibroblast is due to competitive depletion of 9-cis RA (mediated via RAR-binding).

The promiscuity of RXR when forming heterodimers with other nuclear receptors warrants further studies to elucidate the interplay between different steroid nuclear receptors (Hyter and Indra 2013). In particular, overlapping of the vitamin D and retinoic acid (RA) signalling pathways and their convergence at a common response element has been reported (Tavera-Mendoza et al. 2006). Competition for this shared DNA sequence of the two overlapping steroid response elements has been demonstrated by Cao *et al* (1996); with retinoic acid blunting vitamin D_3-mediated integrin transcription as a result (Cao et al. 1996). Similarly, vitamin D_3 was also found to reduce the biological effects of RA via transcriptional interference of activated VDR on the retinoid acid response element (Jiménez-Lara and Aranda 1999). To establish the affinity of the VDR-RXR and RAR-RXR complexes for their respective response elements in dermal fibroblasts, Scatchard analysis can be performed as previously described (Schräder et al. 1994; Cao et al. 1996). This can be followed by alternative knockdown of VDR and RAR, while in the presence of 1,25$(OH)_2D_3$,

to determine whether there is any difference in phenotypic changes (e.g. cells migration and proliferation) observed.

Lastly, to translate the results of this study into changes in clinical practice, the first step would be to determine whether there is a correlation between low serum level of vitamin D_3 with the prevalence of chronic wounds and abnormal scarring (e.g. keloid and hypertrophic scarring) among burns patients. If a correlation is found, a double-blinded randomized controlled trial can be designed with vitamin D_3-deficient patients being allocated into the treatment (oral vitamin D_3 supplement) or placebo groups. Prospective data can then be collected to determine whether oral vitamin D_3 supplements produce any changes in the wound healing or scarring response. A cohort study can also be set up to establish the therapeutic differences between oral vitamin D_3 supplement and topical application of vitamin D_3 in chronic wound and abnormal scar. However, more *in vitro* and *in vivo* studies using models such as *ex vivo* wound healing assay and collagen gel assays are required to provide preliminary data prior to application in clinical trials. This is especially important among groups of patients that are not vitamin D_3-deficient since this may raise concerns regarding possible vitamin D_3 toxicity.

7 ORAL PRESENTATIONS AND AWARDS

7.1 ORAL PRESENTATIONS

a) J.Q.Tay, A.M. Graham, A.L. Mahajan, M.J. Thornton. *Different roles of vitamin D_3 in human skin, regulation of matrix metalloproteinase-2, alpha-smooth muscle actin and collagen I and III: implications for wound healing and scarring*. European Association of Plastic Surgeons Annual Meeting, **2018**, Madrid, Spain.

b) J.Q. Tay, A.M. Graham, A.L. Mahajan, M.J. Thornton. *Role of vitamin D in cutaneous wound healing: different effects on human epidermal keratinocytes and dermal fibroblasts*. European Association of Plastic Surgeons Annual Meeting, **2017**, Milan, Italy.

c) J.Q. Tay, O. Kamala, A.M. Graham, M.J. Thornton, A.L. Mahajan. *Regulation of vitamin D bioavailability in human skin by fibroblasts and keratinocytes during wound healing*. British Association of Plastic, Reconstructive and Aesthetic Surgeons Winter Scientific Meeting, **2016**, London, United Kingdom.

d) J.Q. Tay, O. Kamala, A.M. Graham, M.J. Thornton, A.L. Mahajan. *Intracrine and paracrine regulation of biologically active vitamin D by human dermal fibroblasts: implications for cutaneous wound healing*. British Association of Plastic, Reconstructive and Aesthetic Surgeons Winter Scientific Meeting, **2015**, London, United Kingdom.

e) J.Q. Tay, O. Kamala, A.M. Graham, A.L. Mahajan, M.J. Thornton. *The role of human dermal fibroblasts in regulating availability of biologically active vitamin D: implications for cutaneous wound healing.* 7th Joint Meeting of the European Tissue Repair Society and the Wound Healing Society, **2015**, Copenhagen, Denmark.

7.2 POSTER PRESENTATIONS

a) J.Q. Tay, A.M. Graham, A.L. Mahajan, M.J. Thornton. 2018. *1,25-dihydroxyvitamin D_3 downregulates matrix metalloproteinases-2 and alpha-smooth muscle actin in wounded human dermal fibroblasts: implications for scarring*. British Society for Investigative Dermatology Annual Meeting, **2018**, London, United Kingdom.

b) J.Q. Tay, A.M. Graham, A.L. Mahajan, M.J. Thornton. 2017. *1,25-dihydroxyvitamin D_3 stimulates human epidermal wound closure and reduces dermal fibroblast α-SMA expression*. British Society for Investigative Dermatology Annual Meeting, **2017**, London, United Kingdom.

7.3 AWARDS

a) **Best paper in EURAPS Research Council presentations**. European Association of Plastic Surgeons Annual Meeting, **2018**, Madrid, Spain.

b) **Best poster presentation**. British Society of Investigative Dermatology Annual Meeting, **2018**, London, United Kingdom.

8 REFERENCES

Abe, M., Kurosawa, M., Igarashi, Y., Ishikawa, O. and Miyachi, Y. (2000) Influence of IgE-mediated activation of cultured human mast cells on proliferation and type I collagen production by human dermal fibroblasts. *Journal of allergy and clinical immunology* 106 (1), S72-S77.

Adiri, R. (2009) *Vitamin D accelerates epidermal keratinocyte migration.* ies.org.il: Illuminating Engineering Society. http://www.ies.org.il/abstracts09/bone%20metabolisim%20and%20vit%20D%20oral15-21.pdf Accessed 31 May 2016.

Ahn, J., Yu, K., Stolzenberg-Solomon, R., Simon, K. C., McCullough, M. L., Gallicchio, L., Jacobs, E. J., Ascherio, A., Helzlsouer, K. and Jacobs, K. B. (2010) Genome-wide association study of circulating vitamin D levels. *Human molecular genetics*, ddq155.

Aiba, I., Yamasaki, T., Shinki, T., Izumi, S., Yamamoto, K., Yamada, S., Terato, H., Ide, H. and Ohyama, Y. (2006) Characterization of rat and human CYP2J enzymes as Vitamin D 25-hydroxylases. *Steroids* 71 (10), 849-56.

Akner, G., Wikström, A.-C. and Gustafsson, J.-Å. (1995) Subcellular distribution of the glucocorticoid receptor and evidence for its association with microtubules. *The Journal of steroid biochemistry and molecular biology* 52 (1), 1-16.

Al-Nasiry, S., Geusens, N., Hanssens, M., Luyten, C. and Pijnenborg, R. (2007) The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. *Human Reproduction* 22 (5), 1304-1309.

Alaiti, S. (2015) *Diagrammatic Representatin of Hair Anatomy.* The University of British Columbia. http://www.dermweb.com/hairnailsmucousmembranes/diagram3page.htm Accessed 01 April.

Alaiti, S. (2015) *Hair Anatomy.* Medscape. http://emedicine.medscape.com/article/835470-overview Accessed 01 April.

Ali Bahar, M., Bauer, B., Tredget, E. E. and Ghahary, A. (2004) Dermal fibroblasts from different layers of human skin are heterogeneous in expression of collagenase and types I and III procollagen mRNA. *Wound repair and regeneration* 12 (2), 175-182.

Alonso, L. and Fuchs, E. (2003) Stem cells of the skin epithelium. *Proceedings of the National Academy of Sciences* 100 (suppl 1), 11830-11835.

Amento, E. P. (1987) Vitamin D and the immune system. *Steroids* 49 (1-3), 55-72.

Amini-Nik, S., Cambridge, E., Yu, W., Guo, A., Whetstone, H., Nadesan, P., Poon, R., Hinz, B. and Alman, B. A. (2014) β-Catenin–regulated myeloid cell adhesion and migration determine wound healing. *The Journal of Clinical Investigation* 124 (6), 2599-2610.

Anderson, P. H., May, B. K. and Morris, H. A. (2003) Vitamin D Metabolism: New Concepts and Clinical Implications. *The Clinical Biochemist Reviews* 24 (1), 13-26.

Aranow, C. (2011) Vitamin D and the Immune System. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research* 59 (6), 881-886.

Armas, L. A., Hollis, B. W. and Heaney, R. P. (2004) Vitamin D2 is much less effective than vitamin D3 in humans. *J Clin Endocrinol Metab* 89 (11), 5387-91.

Aslam, F., McCabe, L., Frenkel, B., van Wijnen, A. J., Stein, G. S., Lian, J. B. and Stein, J. L. (1999) AP-1 and vitamin D receptor (VDR) signaling pathways converge at the rat osteocalcin VDR element: requirement for the internal activating protein-1 site for vitamin D-mediated trans-activation. *Endocrinology* 140 (1), 63-70.

Atkinson, J. J., Toennies, H. M., Holmbeck, K. and Senior, R. M. (2007) Membrane type 1 matrix metalloproteinase is necessary for distal airway epithelial repair and keratinocyte growth factor receptor expression after acute injury. *Am J Physiol Lung Cell Mol Physiol* 293 (3), L600-10.

Autier, P., Boniol, M., Pizot, C. and Mullie, P. (2014) Vitamin D status and ill health: a systematic review. *The lancet Diabetes & endocrinology* 2 (1), 76-89.

Bailey, S. J. (2010) Vulnerability of the Brain to Neuropsychiatric Disorders Resulting from Abnormal Thyroid Hormone or Vitamin D Homeostasis. In M.S., R. (editor) *Brain Protection in Schizophrenia, Mood and Cognitive Disorders.* 105-134.

Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H. and Tomic-Canic, M. (2008) Growth factors and cytokines in wound healing. *Wound Repair Regen* 16 (5), 585-601.

Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H. and Tomic-Canic, M. (2008) Growth factors and cytokines in wound healing. *Wound repair and regeneration* 16 (5), 585-601.

Barsony, J. and McKoy, W. (1992) Molybdate increases intracellular 3', 5'-guanosine cyclic monophosphate and stabilizes vitamin D receptor association with tubulin-containing filaments. *Journal of Biological Chemistry* 267 (34), 24457-24465.

Barsony, J., Renyi, I. and McKoy, W. (1997) Subcellular Distribution of Normal and Mutant Vitamin D Receptors in Living Cells STUDIES WITH A NOVEL FLUORESCENT LIGAND. *Journal of Biological Chemistry* 272 (9), 5774-5782.

Battegay, E. J., Raines, E. W., Colbert, T. and Ross, R. (1995) TNF-alpha stimulation of fibroblast proliferation. Dependence on platelet-derived growth factor (PDGF) secretion and alteration of PDGF receptor expression. *J Immunol* 154 (11), 6040-7.

Beckman, M. J. and Deluca, H. F. (1998) 1 - Modern View of Vitamin D3 and its Medicinal Uses. In Ellis, G. P., Luscombe, D. K., and Oxford, A. W. (editors) *Progress in Medicinal Chemistry.* Vol. 35. Elsevier. 1-56.

Behm, B., Babilas, P., Landthaler, M. and Schreml, S. (2012) Cytokines, chemokines and growth factors in wound healing. *J Eur Acad Dermatol Venereol* 26 (7), 812-20.

Bendik, I., Friedel, A., Roos, F. F., Weber, P. and Eggersdorfer, M. (2014) Vitamin D: a critical and essential micronutrient for human health. *Frontiers in Physiology* 5, 14.

Betsuyaku, T., Fukuda, Y., Parks, W. C., Shipley, J. M. and Senior, R. M. (2000) Gelatinase B is required for alveolar bronchiolization after intratracheal bleomycin. *Am J Pathol* 157 (2), 525-35.

Bian, Y. S., Osterheld, M. C., Bosman, F. T., Fontolliet, C. and Benhattar, J. (2000) Nuclear accumulation of beta-catenin is a common and early event during neoplastic progression of Barrett esophagus. *Am J Clin Pathol* 114 (4), 583-90.

Bikle, D. (2017) *Vitamin D: Production, Metabolism, and Mechanism of Action.* 11 August 2017. South Dartmouth: Endotext. Accessed 19/04/2018.

Bikle, D. D. (1982) The vitamin D endocrine system. *Adv Intern Med* 27, 45-71.

Bikle, D. D. (2011) Vitamin D Metabolism and Function in the Skin. *Molecular and cellular endocrinology* 347 (1-2), 80-89.

Bikle, D. D., Tu, C.-L. and Oda, Y. (2014) Calcium and Vitamin D Signaling in the Epidermal Response to Wounding. *Immunology Endocrine & Metabolic Agents in Medicinal Chemistry* 14 (3), 128-136.

Blair, P. M. (2012) Action needed on vitamin D levels. *BBC News*, sec. Health, *http://www.bbc.co.uk/news/health-20710026* Accessed 02 April 2016

Bogatkevich, G. S. (2015) Editorial: Fate of fat tissue adipocytes: do they transform into myofibroblasts in scleroderma? *Arthritis Rheumatol* 67 (4), 860-1.

Bouillon, R., Carmeliet, G., Verlinden, L., van Etten, E., Verstuyf, A., Luderer, H. F., Lieben, L., Mathieu, C. and Demay, M. (2008) Vitamin D and Human Health: Lessons from Vitamin D Receptor Null Mice. *Endocrine Reviews* 29 (6), 726-776.

Bouillon, R., Okamura, W. H. and Norman, A. W. (1995) Structure-function relationships in the vitamin D endocrine system. *Endocr Rev* 16 (2), 200-57.

Boulais, N. and Misery, L. (2008) The epidermis: a sensory tissue. *Eur J Dermatol* 18 (2), 119-27.

Boxman, I., Lowik, C., Aarden, L. and Ponec, M. (1993) Modulation of IL-6 production and IL-1 activity by keratinocyte-fibroblast interaction. *J Invest Dermatol* 101 (3), 316-24.

Bragulla, H. H. and Homberger, D. G. (2009) Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. *Journal of Anatomy* 214 (4), 516-559.

Braun-Falco, G. P., H. H. Wolff (1996) *Dermatology.* Berlin: Springer.

Breitkreutz, D., Mirancea, N. and Nischt, R. (2009) Basement membranes in skin: unique matrix structures with diverse functions? *Histochemistry and Cell Biology* 132 (1), 1-10.

Brenner, M. and Hearing, V. J. (2008) The Protective Role of Melanin Against UV Damage in Human Skin. *Photochemistry and photobiology* 84 (3), 539-549.

Breuhahn, K., Mann, A., Müller, G., Wilhelmi, A., Schirmacher, P., Enk, A. and Blessing, M. (2000) Epidermal overexpression of granulocyte-macrophage colony-stimulating factor induces both keratinocyte proliferation and apoptosis. *Cell growth & differentiation: the molecular biology journal of the American Association for Cancer Research* 11 (2), 111-121.

Brewer, L. D., Thibault, V., Chen, K. C., Langub, M. C., Landfield, P. W. and Porter, N. M. (2001) Vitamin D hormone confers neuroprotection in parallel with downregulation of L-type calcium channel expression in hippocampal neurons. *J Neurosci* 21 (1), 98-108.

Brink, H. E., Stalling, S. S. and Nicoll, S. B. (2005) Influence of serum on adult and fetal dermal fibroblast migration, adhesion, and collagen expression. *In Vitro Cellular & Developmental Biology-Animal* 41 (8), 252-257.

Bruch-Gerharz, D., Ruzicka, T. and Kolb-Bachofen, V. (1998) Nitric oxide and its implications in skin homeostasis and disease - a review. *Arch Dermatol Res* 290 (12), 643-51.

Bugge, T. H., Pohl, J., Lonnoy, O. and Stunnenberg, H. G. (1992) RXR alpha, a promiscuous partner of retinoic acid and thyroid hormone receptors. *Embo j* 11 (4), 1409-18.

Buitrago, C., Vazquez, G., De Boland, A. R. and Boland, R. (2001) The Vitamin D Receptor Mediates Rapid Changes in Muscle Protein Tyrosine Phosphorylation Induced by 1,25(OH)2D3. *Biochemical and Biophysical Research Communications* 289 (5), 1150-1156.

Buitrago, C., Vazquez, G., De Boland, A. R. and Boland, R. L. (2000) Activation of Src kinase in skeletal muscle cells by 1, 1,25-(OH(2))-vitamin D(3) correlates with tyrosine phosphorylation of the vitamin D receptor (VDR) and VDR-Src interaction. *J Cell Biochem* 79 (2), 274-81.

Bullard, K. M., Lund, L., Mudgett, J. S., Mellin, T. N., Hunt, T. K., Murphy, B., Ronan, J., Werb, Z. and Banda, M. J. (1999) Impaired wound contraction in stromelysin-1-deficient mice. *Ann Surg* 230 (2), 260-5.

Burkiewicz, C. J., Guadagnin, F. A., Skare, T. L., do Nascimento, M. M., Servin, S. C. and de Souza, G. D. (2012) Vitamin D and skin repair: a prospective, double-blind and placebo controlled study in the healing of leg ulcers. *Rev Col Bras Cir* 39 (5), 401-7.

Burkiewicz, C. J. C. C., Guadagnin, F. A., Skare, T. L., Nascimento, M. M. d., Servin, S. C. N. and Souza, G. D. d. (2012) Vitamina D e cicatrização de pele: estudo prospectivo, duplo-cego, placebo controlado na cicatrização de úlceras de perna. *Revista do Colégio Brasileiro de Cirurgiões* 39, 401-407.

Burkitt H. G., Y. B. (1993) *Wheater's Functional Histology.* 3rd Ed edition. New York: Livingstone.

Caley, M. P., Martins, V. L. C. and O'Toole, E. A. (2015) Metalloproteinases and Wound Healing. *Advances in Wound Care* 4 (4), 225-234.

Cao, X., Teitelbaum, S. L., Zhu, H.-J., Zhang, L., Feng, X. and Ross, F. P. (1996) Competition for a unique response element mediates retinoic acid inhibition of vitamin D3-stimulated transcription. *Journal of Biological Chemistry* 271 (34), 20650-20654.

Cao, X., Teitelbaum, S. L., Zhu, H. J., Zhang, L., Feng, X. and Ross, F. P. (1996) Competition for a unique response element mediates retinoic acid inhibition of vitamin D3-stimulated transcription. *J Biol Chem* 271 (34), 20650-4.

Carlberg, C. and Campbell, M. J. (2013) Vitamin D receptor signaling mechanisms: integrated actions of a well-defined transcription factor. *Steroids* 78 (2), 127-136.

Carlberg, C. and Seuter, S. (2009) A genomic perspective on vitamin D signaling. *Anticancer research* 29 (9), 3485-3493.

Cashman, K. D., Dowling, K. G., Škrabáková, Z., Gonzalez-Gross, M., Valtueña, J., De Henauw, S., Moreno, L., Damsgaard, C. T., Michaelsen, K. F. and Mølgaard, C. (2016) Vitamin D deficiency in Europe: pandemic? *The American journal of clinical nutrition*, ajcn120873.

Chakrabarti, S. and Patel, K. D. (2005) Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. *Exp Lung Res* 31 (6), 599-621.

Chawla, A., Repa, J. J., Evans, R. M. and Mangelsdorf, D. J. (2001) Nuclear receptors and lipid physiology: opening the X-files. *Science* 294 (5548), 1866-70.

Chen, C. H., Sakai, Y. and Demay, M. B. (2001) Targeting expression of the human vitamin D receptor to the keratinocytes of vitamin D receptor null mice prevents alopecia. *Endocrinology* 142 (12), 5386-9.

Chen, H. C. and Farese, R. V. (1999) Steroid hormones: Interactions with membrane-bound receptors. *Current Biology* 9 (13), R478-R481.

Chen, P., Abacherli, L. E., Nadler, S. T., Wang, Y., Li, Q. and Parks, W. C. (2009) MMP7 shedding of syndecan-1 facilitates re-epithelialization by affecting alpha(2)beta(1) integrin activation. *PLoS One* 4 (8), e6565.

Chen, P. and Parks, W. C. (2009) Role of matrix metalloproteinases in epithelial migration. *Journal of Cellular Biochemistry* 108 (6), 1233-1243.

Chen, T. C., Persons, K. S., Lu, Z., Mathieu, J. S. and Holick, M. F. (2000) An evaluation of the biologic activity and vitamin D receptor binding affinity of the photoisomers of vitamin D3 and previtamin D3. *J Nutr Biochem* 11 (5), 267-72.

Cheng, J. B., Levine, M. A., Bell, N. H., Mangelsdorf, D. J. and Russell, D. W. (2004) Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proceedings of the National Academy of Sciences of the United States of America* 101 (20), 7711-7715.

Cheng, W., Yan-hua, R., Fang-gang, N. and Guo-an, Z. (2011) The content and ratio of type I and III collagen in skin differ with age and injury. *African Journal of Biotechnology* 10 (13), 2524-2529.

Chitturi, R. T., Balasubramaniam, A. M., Parameswar, R. A., Kesavan, G., Haris, K. T. M. and Mohideen, K. (2015) The Role of Myofibroblasts in Wound Healing, Contraction and its Clinical Implications in Cleft Palate Repair. *Journal of International Oral Health : JIOH* 7 (3), 75-80.

Choi, M., Yamada, S. and Makishima, M. (2011) Dynamic and ligand-selective interactions of vitamin D receptor with retinoid X receptor and cofactors in living cells. *Mol Pharmacol* 80 (6), 1147-55.

Chomiczewska, D., Trznadel-Budzko, E., Kaczorowska, A. and Rotsztejn, H. (2009) [The role of Langerhans cells in the skin immune system]. *Pol Merkur Lekarski* 26 (153), 173-7.

Christakos, S., Ajibade, D. V., Dhawan, P., Fechner, A. J. and Mady, L. J. (2010) Vitamin D: Metabolism. *Endocrinology and metabolism clinics of North America* 39 (2), 243-253.

Chun, R. F., Peercy, B. E., Orwoll, E. S., Nielson, C. M., Adams, J. S. and Hewison, M. (2014) Vitamin D and DBP: The free hormone hypothesis revisited. *The Journal of steroid biochemistry and molecular biology* 144PA, 132-137.

Chung, J. H., Seo, J. Y., Choi, H. R., Lee, M. K., Youn, C. S., Rhie, G.-e., Cho, K. H., Kim, K. H., Park, K. C. and Eun, H. C. (2001) Modulation of Skin Collagen Metabolism in Aged and Photoaged Human Skin In Vivo. *Journal of Investigative Dermatology* 117 (5), 1218-1224.

Chunmeng, S. and Tianmin, C. (2004) Skin: a promising reservoir for adult stem cell populations. *Med Hypotheses* 62 (5), 683-8.

Cianferotti, L., Cox, M., Skorija, K. and Demay, M. B. (2007) Vitamin D receptor is essential for normal keratinocyte stem cell function. *Proceedings of the National Academy of Sciences* 104 (22), 9428-9433.

Coelho, M., Oliveira, T. and Fernandes, R. (2013) Biochemistry of adipose tissue: an endocrine organ. *Archives of Medical Science : AMS* 9 (2), 191-200.

Costa-Almeida, R., Gomez-Lazaro, M., Ramalho, C., Granja, P. L., Soares, R. and Guerreiro, S. G. (2015) Fibroblast-Endothelial Partners for Vascularization Strategies in Tissue Engineering. *Tissue Engineering. Part A* 21 (5-6), 1055-1065.

D'Orazio, J., Jarrett, S., Amaro-Ortiz, A. and Scott, T. (2013) UV Radiation and the Skin. *International Journal of Molecular Sciences* 14 (6), 12222-12248.

Dahiru, T. (2008) P – VALUE, A TRUE TEST OF STATISTICAL SIGNIFICANCE? A CAUTIONARY NOTE. *Annals of Ibadan Postgraduate Medicine* 6 (1), 21-26.

Darby, I. A., Laverdet, B., Bonté, F. and Desmoulière, A. (2014) Fibroblasts and myofibroblasts in wound healing. *Clinical, Cosmetic and Investigational Dermatology* 7, 301-311.

Darley, C., Cunliffe, W., Green, C., Hutchinson, P., Klaber, M. and Downes, N. (1996) Safety and efficacy of calcipotriol ointment (Dovonex®) in treating children with psoriasis vulgaris. *British Journal of Dermatology* 135 (3), 390-393.

Dawson, M. I. and Xia, Z. (2012) The Retinoid X Receptors and Their Ligands. *Biochimica et biophysica acta* 1821 (1), 21-56.

De Haes, P., Garmyn, M., Degreef, H., Vantieghem, K., Bouillon, R. and Segaert, S. (2003) 1, 25-dihydroxyvitamin D3 inhibits ultraviolet B-induced apoptosis, Jun kinase activation, and interleukin-6 production

in primary human keratinocytes. *Journal of cellular biochemistry* 89 (4), 663-673.
Deeb, K. K., Trump, D. L. and Johnson, C. S. (2007) Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nature Reviews Cancer* 7, 684.
Deeb, K. K., Trump, D. L. and Johnson, C. S. (2007) Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nature Reviews Cancer* 7 (9), 684-700.
DeLuca, H. F. (2014) History of the discovery of vitamin D and its active metabolites. *BoneKEy reports* 3.
Demay, M. B. (2012) The hair cycle and Vitamin D receptor. *Arch Biochem Biophys* 523 (1), 19-21.
Di Rosa, M., Malaguarnera, M., Nicoletti, F. and Malaguarnera, L. (2011) Vitamin D3: a helpful immuno-modulator. *Immunology* 134 (2), 123-139.
Díaz, L., Díaz-Muñoz, M., García-Gaytán, A. C. and Méndez, I. (2015) Mechanistic Effects of Calcitriol in Cancer Biology. *Nutrients* 7 (6), 5020-5050.
Dickson, I. R. and Maher, P. M. (1985) The influence of vitamin D metabolites on collagen synthesis by chick cartilage in organ culture. *J Endocrinol* 105 (1), 79-85.
DiMartino, S. J., Trujillo, G., McVoy, L. A., Zhang, J. and Kew, R. R. (2007) Upregulation of Vitamin D Binding Protein (Gc-Globulin) Binding Sites During Neutrophil Activation from a Latent Reservoir in Azurophil Granules. *Molecular immunology* 44 (9), 2370-2377.
Ding, N., Yu, R. T., Subramaniam, N., Sherman, M. H., Wilson, C., Rao, R., Leblanc, M., Coulter, S., He, M., Scott, C., Lau, S. L., Atkins, A. R., Barish, G. D., Gunton, J. E., Liddle, C., Downes, M. and Evans, R. M. (2013) A Vitamin D Receptor/SMAD Genomic Circuit Gates Hepatic Fibrotic Response. *Cell* 153 (3), 601-613.
Dobak, J., Grzybowski, J., Liu, F.-T., Landon, B. and Dobke, M. (1994) 1,25-Dihydroxyvitamin D3 increases collagen production in dermal fibroblasts. *Journal of Dermatological Science* 8 (1), 18-24.
Dobak, J., Grzybowski, J., Liu, F. T., Landon, B. and Dobke, M. (1994) 1,25-Dihydroxyvitamin D3 increases collagen production in dermal fibroblasts. *J Dermatol Sci* 8 (1), 18-24.
Driskell, R., Jahoda, C. A. B., Chuong, C.-M., Watt, F. and Horsley, V. (2014) Defining dermal adipose tissue. *Experimental dermatology* 23 (9), 629-631.
Driskell, R. R., Clavel, C., Rendl, M. and Watt, F. M. (2011) Hair follicle dermal papilla cells at a glance. *Journal of Cell Science* 124 (8), 1179-1182.
Du, T., Zhou, Z.-G., You, S., Huang, G., Lin, J., Yang, L., Li, X., Zhou, W.-D. and Chao, C. (2009) Modulation of monocyte hyperresponsiveness to TLR ligands by 1, 25-dihydroxy-vitamin D3 from LADA and T2DM. *Diabetes research and clinical practice* 83 (2), 208-214.
Dugina, V., Fontao, L., Chaponnier, C., Vasiliev, J. and Gabbiani, G. (2001) Focal adhesion features during myofibroblastic differentiation are

controlled by intracellular and extracellular factors. *J Cell Sci* 114 (Pt 18), 3285-96.
Dusso, A. S., Brown, A. J. and Slatopolsky, E. (2005) Vitamin D. *American Journal of Physiology - Renal Physiology* 289 (1), F8-F28.
Elangovan, H., Chahal, S. and Gunton, J. E. (2017) Vitamin D in liver disease: Current evidence and potential directions. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1863 (4), 907-916.
Ellfolk, M., Norlin, M., Gyllensten, K. and Wikvall, K. (2009) Regulation of human vitamin D3 25-hydroxylases in dermal fibroblasts and prostate cancer LNCaP cells. *Molecular pharmacology* 75 (6), 1392-1399.
Ellison, T. I., Smith, M. K., Gilliam, A. C. and MacDonald, P. N. (2008) Inactivation of the Vitamin D Receptor Enhances Susceptibility of Murine Skin to UV-induced Tumorigenesis. *The Journal of investigative dermatology* 128 (10), 2508-2517.
Evans, J. H. and Falke, J. J. (2007) Ca(2+) influx is an essential component of the positive-feedback loop that maintains leading-edge structure and activity in macrophages. *Proceedings of the National Academy of Sciences of the United States of America* 104 (41), 16176-16181.
Evans, R. M. (1988) The steroid and thyroid hormone receptor superfamily. *Science* 240 (4854), 889-95.
Ezure, T. and Amano, S. (2011) Negative regulation of dermal fibroblasts by enlarged adipocytes through release of free fatty acids. *J Invest Dermatol* 131 (10), 2004-9.
Falke, L. L., Gholizadeh, S., Goldschmeding, R., Kok, R. J. and Nguyen, T. Q. (2015) Diverse origins of the myofibroblast—implications for kidney fibrosis. *Nature Reviews Nephrology* 11, 233.
Farach-Carson, M. C. and Nemere, I. (2003) Membrane receptors for vitamin D steroid hormones: potential new drug targets. *Curr Drug Targets* 4 (1), 67-76.
Feldman, D. (2011) Vitamin D Metabolism *Vitamin D.*Vitamin D. Vol. One. Third Edition edition. American Press.
Feldman, D., CHEN, T., HIRST, M., COLSTON, K., KARASEK, M. and CONE, C. (1980) Demonstration of 1, 25-dihydroxyvitamin D3 receptors in human skin biopsies. *The Journal of Clinical Endocrinology & Metabolism* 51 (6), 1463-1465.
Feldman, D. and Malloy, P. (2014) Mutations in the vitamin D receptor and hereditary vitamin D-resistant rickets. *BoneKEy Rep* 3.
Fleet, J. C. (2004) Rapid, Membrane-Initiated Actions of 1,25 Dihydroxyvitamin D: What Are They and What Do They Mean? *The Journal of Nutrition* 134 (12), 3215-3218.
Fleet, J. C. (2004) Rapid, membrane-initiated actions of 1,25 dihydroxyvitamin D: what are they and what do they mean? *J Nutr* 134 (12), 3215-8.
Fleischmajer, R., Kühn, K., Sato, Y., Douglas MacDonald Ii, E., Perlish, J. S., Pan, T.-C., Chu, M.-L., Kishiro, Y., Oohashi, T., Bernier, S. M., Yamada, Y. and Ninomiya, Y. (1997) There Is Temporal and Spatial Expression of α1 (IV), α2 (IV), α5 (IV), α6 (IV) Collagen Chains and β1 Integrins During

the Development of the Basal Lamina in an “In Vitro” Skin Model. *Journal of Investigative Dermatology* 109 (4), 527-533.

Fleischmajer, R., Utani, A., MacDonald Ii, E. D., Perlish, J. S., Pan, T. C., Chu, M. L., Nomizu, M., Ninomiya, Y. and Yamada, Y. (1998) Initiation of skin basement membrane formation at the epidermo-dermal interface involves assembly of laminins through binding to cell membrane receptors. *Journal of Cell Science* 111 (14), 1929-1940.

Franceschi, R., Romano, P. and Park, K. (1988) Regulation of type I collagen synthesis by 1, 25-dihydroxyvitamin D3 in human osteosarcoma cells. *Journal of Biological Chemistry* 263 (35), 18938-18945.

Franceschi, R. T. and Iyer, B. S. (1992) Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *Journal of Bone and Mineral Research* 7 (2), 235-246.

Frazier, K., Williams, S., Kothapalli, D., Klapper, H. and Grotendorst, G. R. (1996) Stimulation of Fibroblast Cell Growth, Matrix Production, and Granulation Tissue Formation by Connective Tissue Growth Factor. *Journal of Investigative Dermatology* 107 (3), 404-411.

Fuchs, E. (2008) Skin stem cells: rising to the surface. *The Journal of cell biology* 180 (2), 273-284.

Gabbiani, G. (2003) The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 200 (4), 500-3.

Gabbiani, G., Ryan, G. B. and Majne, G. (1971) Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 27 (5), 549-50.

Gambichler, T., Bindsteiner, M., Hoxtermann, S. and Kreuter, A. (2013) Serum 25-hydroxyvitamin D serum levels in a large German cohort of patients with melanoma. *Br J Dermatol* 168 (3), 625-8.

Gangnuss, S., Cowin, A. J., Daehn, I. S., Hatzirodos, N., Rothnagel, J. A., Varelias, A. and Rayner, T. E. (2004) Regulation of MAPK activation, AP-1 transcription factor expression and keratinocyte differentiation in wounded fetal skin. *J Invest Dermatol* 122 (3), 791-804.

Gangnuss, S., Cowin, A. J., Daehn, I. S., Hatzirodos, N., Rothnagel, J. A., Varelias, A. and Rayner, T. E. (2004) Regulation of MAPK Activation, AP-1 Transcription Factor Expression and Keratinocyte Differentiation in Wounded Fetal Skin. *Journal of Investigative Dermatology* 122 (3), 791-804.

Garlick, J. A. and Taichman, L. B. (1994) FATE OF HUMAN KERATINOCYTES DURING REEPITHELIALIZATION IN AN ORGANOTYPIC CULTURE MODEL. *Laboratory Investigation* 70 (6), 916-924.

Garner, W. L. (1998) Epidermal regulation of dermal fibroblast activity. *Plast Reconstr Surg* 102 (1), 135-9.

Garza, L. A., Yang, C.-C., Zhao, T., Blatt, H. B., Lee, M., He, H., Stanton, D. C., Carrasco, L., Spiegel, J. H., Tobias, J. W. and Cotsarelis, G. (2011) Bald scalp in men with androgenetic alopecia retains hair follicle stem cells but lacks CD200-rich and CD34-positive hair follicle progenitor cells. *The Journal of Clinical Investigation* 121 (2), 613-622.

Gavet, O. and Pines, J. (2010) Activation of cyclin B1–Cdk1 synchronizes events in the nucleus and the cytoplasm at mitosis. *The Journal of cell biology* 189 (2), 247-259.

Gay, R. E., Buckingham, R. B., Prince, R. K., Gay, S., Rodnan, G. P. and Miller, E. J. (1980) Collagen types synthesized in dermal fibroblast cultures from patients with early progressive systemic sclerosis. *Arthritis & Rheumatology* 23 (2), 190-196.

Gaylor, J. and Sault, F. M. (1964) Localization and biosynthesis of 7-dehydrocholesterol in rat skin. *Journal of Lipid Research* 5 (3), 422-431.

Gezen-Ak, D., Dursun, E. and Yilmazer, S. (2011) The effects of vitamin D receptor silencing on the expression of LVSCC-A1C and LVSCC-A1D and the release of NGF in cortical neurons. *PLoS One* 6 (3), e17553.

Giannelli, G., Falk-Marzillier, J., Schiraldi, O., Stetler-Stevenson, W. G. and Quaranta, V. (1997) Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science* 277 (5323), 225-8.

Gilchrest, B. A., Karassik, R. L., Wilkins, L. M., Vrabel, M. A. and Maciag, T. (1983) Autocrine and paracrine growth stimulation of cells derived from human skin. *J Cell Physiol* 117 (2), 235-40.

Gill, R. K., Atkins, L. M., Hollis, B. W. and Bell, N. H. (1998) Mapping the domains of the interaction of the vitamin D receptor and steroid receptor coactivator-1. *Molecular Endocrinology* 12 (1), 57-65.

Gipson, I. K., Spurrmichaud, S. J. and Tisdale, A. S. (1988) HEMIDESMOSOMES AND ANCHORING FIBRIL COLLAGEN APPEAR SYNCHRONOUSLY DURING DEVELOPMENT AND WOUND-HEALING. *Developmental Biology* 126 (2), 253-262.

Girgis, C. M., Clifton-Bligh, R. J., Mokbel, N., Cheng, K. and Gunton, J. E. (2014) Vitamin D Signaling Regulates Proliferation, Differentiation, and Myotube Size in C2C12 Skeletal Muscle Cells. *Endocrinology* 155 (2), 347-357.

Gniadecki, R. (1996) Stimulation versus inhibition of keratinocyte growth by 1,25-Dihydroxyvitamin D3: dependence on cell culture conditions. *J Invest Dermatol* 106 (3), 510-6.

Gocek, E., Marchwicka, A., Bujko, K. and Marcinkowska, E. (2014) NADPH-cytochrome p450 reductase is regulated by all-trans retinoic acid and by 1, 25-dihydroxyvitamin D3 in human acute myeloid leukemia cells. *PloS one* 9 (3), e91752.

Goffin, J. M., Pittet, P., Csucs, G., Lussi, J. W., Meister, J. J. and Hinz, B. (2006) Focal adhesion size controls tension-dependent recruitment of alpha-smooth muscle actin to stress fibers. *J Cell Biol* 172 (2), 259-68.

Goldberg, M. T., Han, Y. P., Yan, C., Shaw, M. C. and Garner, W. L. (2007) TNF-alpha suppresses alpha-smooth muscle actin expression in human dermal fibroblasts: an implication for abnormal wound healing. *J Invest Dermatol* 127 (11), 2645-55.

Goldsmith (1991) *Physiology, biochemistry and molecular biology of the skin.* New York: Oxford University Press.

Golebiewska, E. M. and Poole, A. W. (2015) Platelet secretion: From haemostasis to wound healing and beyond. *Blood Reviews* 29 (3), 153-162.

Gombart, A. F. (2009) The vitamin D–antimicrobial peptide pathway and its role in protection against infection. *Future microbiology* 4, 1151.

Gombart, A. F., Borregaard, N. and Koeffler, H. P. (2005) Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *Faseb j* 19 (9), 1067-77.

Gorman, S., Weeden, C. E., Tan, D. H., Scott, N. M., Hart, J., Foong, R. E., Mok, D., Stephens, N., Zosky, G. and Hart, P. H. (2013) Reversible control by vitamin D of granulocytes and bacteria in the lungs of mice: an ovalbumin-induced model of allergic airway disease. *PLoS One* 8 (6), e67823.

Greenbaum, D., Colangelo, C., Williams, K. and Gerstein, M. (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biology* 4 (9), 117-117.

Guillaume (2012) *The dermal-epidermal junction.* *http://biologiedelapeau.fr/spip.php?page=forum&id_article=18&id_forum=213&lang=en* Accessed 30 March.

Gupta, A., Avci, P., Dai, T., Huang, Y.-Y. and Hamblin, M. (2013) *Ultraviolet Radiation in Wound Care: Sterilization and Stimulation.* Vol. 2.

Gupta, R., Dixon, K. M., Deo, S. S., Holliday, C. J., Slater, M., Halliday, G. M., Reeve, V. E. and Mason, R. S. (2007) Photoprotection by 1, 25 dihydroxyvitamin D3 is associated with an increase in p53 and a decrease in nitric oxide products. *Journal of investigative dermatology* 127 (3), 707-715.

Gupta, R. P., He, Y. A., Patrick, K. S., Halpert, J. R. and Bell, N. H. (2005) CYP3A4 is a vitamin D-24- and 25-hydroxylase: analysis of structure function by site-directed mutagenesis. *J Clin Endocrinol Metab* 90 (2), 1210-9.

Gupta, R. P., Hollis, B. W., Patel, S. B., Patrick, K. S. and Bell, N. H. (2004) CYP3A4 is a human microsomal vitamin D 25-hydroxylase. *J Bone Miner Res* 19 (4), 680-8.

Gurtner, G. C., Werner, S., Barrandon, Y. and Longaker, M. T. (2008) Wound repair and regeneration. *Nature* 453 (7193), 314-321.

Guryev, O., Carvalho, R. A., Usanov, S., Gilep, A. and Estabrook, R. W. (2003) A pathway for the metabolism of vitamin D3: unique hydroxylated metabolites formed during catalysis with cytochrome P450scc (CYP11A1). *Proc Natl Acad Sci U S A* 100 (25), 14754-9.

Guyton, A. C. and Hall, J. E. (1996) *Textbook of medical physiology.* 9th ed. edition. Philadelphia: Saunders.

Halloy, J., Bernard, B. A., Loussouarn, G. and Goldbeter, A. (2000) Modeling the dynamics of human hair cycles by a follicular automaton. *Proc Natl Acad Sci U S A* 97 (15), 8328-33.

Halloy, J., Bernard, B. A., Loussouarn, G. and Goldbeter, A. (2002) The follicular automaton model: effect of stochasticity and of synchronization of hair cycles. *J Theor Biol* 214 (3), 469-79.

Hanada, K., Sawamura, D., Nakano, H. and Hashimoto, I. (1995) Possible role of 1,25-dihydroxyvitamin D3-induced metallothionein in photoprotection against UVB injury in mouse skin and cultured rat keratinocytes. *J Dermatol Sci* 9 (3), 203-8.

Hansen, C. M., Mathiasen, I. S. and Binderup, L. (1996) The anti-proliferative and differentiation-inducing effects of vitamin D analogs are not determined by the binding affinity for the vitamin D receptor alone. *J Investig Dermatol Symp Proc* 1 (1), 44-8.

Hart, A. (2001) Mann-Whitney test is not just a test of medians: differences in spread can be important. *BMJ : British Medical Journal* 323 (7309), 391-393.

Hashizume, T., Xu, Y., Mohutsky, M. A., Alberts, J., Hadden, C., Kalhorn, T. F., Isoherranen, N., Shuhart, M. C. and Thummel, K. E. (2008) Identification of human UDP-glucuronosyltransferases catalyzing hepatic 1alpha,25-dihydroxyvitamin D3 conjugation. *Biochem Pharmacol* 75 (5), 1240-50.

Hata, T. R., Kotol, P., Jackson, M., Nguyen, M., Paik, A., Udall, D., Kanada, K., Yamasaki, K., Alexandrescu, D. and Gallo, R. L. (2008) Administration of oral vitamin D induces cathelicidin production in atopic individuals. *The Journal of allergy and clinical immunology* 122 (4), 829-831.

Hattori, N., Mochizuki, S., Kishi, K., Nakajima, T., Takaishi, H., D'Armiento, J. and Okada, Y. (2009) MMP-13 plays a role in keratinocyte migration, angiogenesis, and contraction in mouse skin wound healing. *Am J Pathol* 175 (2), 533-46.

Haubner, F., Muschter, D., Pohl, F., Schreml, S., Prantl, L. and Gassner, H. G. (2015) A Co-Culture Model of Fibroblasts and Adipose Tissue-Derived Stem Cells Reveals New Insights into Impaired Wound Healing After Radiotherapy. *International Journal of Molecular Sciences* 16 (11), 25947-25958.

Haukipuro, K., Melkko, J., Risteli, L., Kairaluoma, M. and Risteli, J. (1991) Synthesis of type I collagen in healing wounds in humans. *Annals of surgery* 213 (1), 75.

Haussler, M. R., Haussler, C. A., Jurutka, P. W., Thompson, P. D., Hsieh, J. C., Remus, L. S., Selznick, S. H. and Whitfield, G. K. (1997) The vitamin D hormone and its nuclear receptor: molecular actions and disease states. *J Endocrinol* 154 Suppl, S57-73.

Haussler, M. R., Whitfield, G. K., Kaneko, I., Haussler, C. A., Hsieh, D., Hsieh, J. C. and Jurutka, P. W. (2013) Molecular mechanisms of vitamin D action. *Calcif Tissue Int* 92 (2), 77-98.

Heidari, F., Yari, A., Rasoolijazi, H., Soleimani, M., Dehpoor, A., Sajedi, N., Joulai Veijouye, S. and Nobakht, M. (2016) Bulge Hair Follicle Stem Cells Accelerate Cutaneous Wound Healing in Rats. *Wounds* 28 (4), 132-41.

Herrmann, M., L Farrell, C.-J., Pusceddu, I., Fabregat-Cabello, N. and Cavalier, E. (2016) *Assessment of Vitamin D status - A changing landscape.* Vol. 55.

Hewison, M., Zehnder, D., Bland, R. and Stewart, P. M. (2000) 1alpha-Hydroxylase and the action of vitamin D. *J Mol Endocrinol* 25 (2), 141-8.

Hieta, N., Impola, U., López-Otín, C., Saarialho-Kere, U. and Kähäri, V.-M. (2003) Matrix metalloproteinase-19 expression in dermal wounds and by fibroblasts in culture. *Journal of investigative dermatology* 121 (5), 997-1004.

Holick, M. F. (2008) Vitamin D and sunlight: strategies for cancer prevention and other health benefits. *Clinical Journal of the American Society of Nephrology* 3 (5), 1548-1554.

Holmes, K., Williams, C. M., Chapman, E. A. and Cross, M. J. (2010) Detection of siRNA induced mRNA silencing by RT-qPCR: considerations for experimental design. *BMC Research Notes* 3, 53-53.

Horne, K. A. and Jahoda, C. A. (1992) Restoration of hair growth by surgical implantation of follicular dermal sheath. *Development* 116 (3), 563-71.

Hosogane, N., Shinki, T., Kasuga, H., Taketomi, S., Toyama, Y. and Suda, T. (2003) Mechanisms for the reduction of 24,25-dihydroxyvitamin D3 levels and bone mass in 24-hydroxylase transgenic rats. *Faseb j* 17 (6), 737-9.

Hosoi, J., Abe, E., Suda, T. and Kuroki, T. (1985) Regulation of melanin synthesis of B16 mouse melanoma cells by 1α, 25-dihydroxyvitamin D3 and retinoic acid. *Cancer Research* 45 (4), 1474-1478.

Huang, P., Chandra, V. and Rastinejad, F. (2013) Retinoic acid actions through mammalian nuclear receptors. *Chemical reviews* 114 (1), 233-254.

Huang, S. J., Lin, C. P. and Tsai, S. Y. (2015) Vitamin D-2 content and antioxidant properties of fruit body and mycelia of edible mushrooms by UV-B irradiation. *Journal of Food Composition and Analysis* 42, 38-45.

Hughes, C. C. (2008) Endothelial-stromal interactions in angiogenesis. *Curr Opin Hematol* 15 (3), 204-9.

Huldschinsky (1919) Heilung von Rachitis durch künstliche Höhensonne. *Dtsch. Med*, 712-713.

Hyppönen, E., Läärä, E., Reunanen, A., Järvelin, M.-R. and Virtanen, S. M. (2001) Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. *The Lancet* 358 (9292), 1500-1503.

Hyter, S. and Indra, A. K. (2013) Nuclear hormone receptor functions in keratinocyte and melanocyte homeostasis, epidermal carcinogenesis and melanomagenesis. *FEBS letters* 587 (6), 529-541.

Imai, Y. and Clemmons, D. R. (1999) Roles of phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways in stimulation of vascular smooth muscle cell migration and deoxyriboncleic acid synthesis by insulin-like growth factor-I. *Endocrinology* 140 (9), 4228-35.

Imai, Y., Youn, M.-Y., Inoue, K., Takada, I., Kouzmenko, A. and Kato, S. (2013) Nuclear receptors in bone physiology and diseases. *Physiological reviews* 93 (2), 481-523.

Inoue, H., Osada, H., Kojima, K., Tomioka, M. and Ishida, H. (1999) Surviving and proliferating faculty of fibroblasts cultured under no-serum and poor

nutritional conditions. The basic study on hybridation of cultured cells with an artificial matrix. *J Cardiovasc Surg (Torino)* 40 (3), 435-8.

Ito, M., Liu, Y., Yang, Z., Nguyen, J., Liang, F., Morris, R. J. and Cotsarelis, G. (2005) Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nature Medicine* 11, 1351.

Jablonski, N. G. and Chaplin, G. (2010) Human skin pigmentation as an adaptation to UV radiation. *Proceedings of the National Academy of Sciences of the United States of America* 107 (Suppl 2), 8962-8968.

Janjetovic, Z., Zmijewski, M. A., Tuckey, R. C., DeLeon, D. A., Nguyen, M. N., Pfeffer, L. M. and Slominski, A. T. (2009) 20-Hydroxycholecalciferol, Product of Vitamin D3 Hydroxylation by P450scc, Decreases NF-κB Activity by Increasing IκBα Levels in Human Keratinocytes. *PLoS ONE* 4 (6), e5988.

Janson, D. G., Saintigny, G., van Adrichem, A., Mahe, C. and El Ghalbzouri, A. (2012) Different gene expression patterns in human papillary and reticular fibroblasts. *J Invest Dermatol* 132 (11), 2565-72.

Jiménez-Lara, A. M. and Aranda, A. (1999) Vitamin D Represses Retinoic Acid-Dependent Transactivation of the Retinoic Acid Receptor-β2 Promoter: The AF-2 Domain of the Vitamin D Receptor Is Required for Transrepression*. *Endocrinology* 140 (6), 2898-2907.

Jimenez, J. J. and Yunis, A. A. (1992) Protection from chemotherapy-induced alopecia by 1,25-dihydroxyvitamin D3. *Cancer Res* 52 (18), 5123-5.

Johri, B. M. (2006) Vitamins: Scurvy, Beri-Beri, Rickets, pellagra, Xerophthalmia, Naming of the Vitamins. In Mehrotra, J. (editor) *History of Biological Science.* New Delhi: Allied Publishers Private Limited. pp. 159-160.

Jones, G. (2008) Pharmacokinetics of vitamin D toxicity. *Am J Clin Nutr* 88 (2), 582s-586s.

Jones, G., Prosser, D. E. and Kaufmann, M. (2014) Thematic Review Series: Fat-Soluble Vitamins: Vitamin D Cytochrome P450-mediated metabolism of vitamin D. *Journal of Lipid Research* 55 (1), 13-31.

Jones, G., Strugnell, S. A. and DeLUCA, H. F. (1998) Current understanding of the molecular actions of vitamin D. *Physiological reviews* 78 (4), 1193-1231.

Kalluri, R. and Neilson, E. G. (2003) Epithelial-mesenchymal transition and its implications for fibrosis. *The Journal of clinical investigation* 112 (12), 1776-1784.

Kamala, O. (2014) *Differences in the expression of inhibitors of apoptosis proteins, oestrogen receptors, and responses to oestradiol under normal and wound induced conditions*. University of Bradford: University of Bradford.

Kamberov, Y. G., Karlsson, E. K., Kamberova, G. L., Lieberman, D. E., Sabeti, P. C., Morgan, B. A. and Tabin, C. J. (2015) A genetic basis of variation in eccrine sweat gland and hair follicle density. *Proceedings of the National Academy of Sciences of the United States of America* 112 (32), 9932-9937.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K. and Rosenfeld, M. G. (1996) A CBP Integrator Complex Mediates Transcriptional Activation and AP-1 Inhibition by Nuclear Receptors. *Cell* 85 (3), 403-414.

Kang, S., Li, X.-Y., Duell, E. A. and Voorhees, J. J. (1997) The Retinoid X Receptor Agonist 9-cis-Retinoic Acid and the 24-Hydroxylase Inhibitor Ketoconazole Increase Activity of 1,25-Dihydroxyvitamin D3 in Human Skin In Vivo. *Journal of Investigative Dermatology* 108 (4), 513-518.

Kanitakis, J. (2002) Anatomy, histology and immunohistochemistry of normal human skin. *Eur J Dermatol* 12 (4), 390-9; quiz 400-1.

Keisala, T., Minasyan, A., Lou, Y.-R., Zou, J., Kalueff, A. V., Pyykkö, I. and Tuohimaa, P. (2009) Premature aging in vitamin D receptor mutant mice. *The Journal of steroid biochemistry and molecular biology* 115 (3-5), 91-97.

Kellner, J. C. and Coulombe, P. A. (2009) Keratins and protein synthesis: the plot thickens. *The Journal of cell biology* 187 (2), 157-159.

Kessler, D., Dethlefsen, S., Haase, I., Plomann, M., Hirche, F., Krieg, T. and Eckes, B. (2001) Fibroblasts in mechanically stressed collagen lattices assume a “synthetic” phenotype. *Journal of Biological Chemistry* 276 (39), 36575-36585.

Khan, M. S., Hryb, D. J., Hashim, G. A., Romas, N. A. and Rosner, W. (1990) Delineation and synthesis of the membrane receptor-binding domain of sex hormone-binding globulin. *J Biol Chem* 265 (30), 18362-5.

Khanal, R. C., Peters, T. M., Smith, N. M. and Nemere, I. (2008) Membrane receptor-initiated signaling in 1,25(OH)2D3-stimulated calcium uptake in intestinal epithelial cells. *J Cell Biochem* 105 (4), 1109-16.

Kidd, R. L., Krawczyk, W. S. and Wilgram, G. F. (1971) The Merkel cell in human epidermis: its differentiation from other dendritic cells. *Archiv für dermatologische Forschung* 241 (4), 374-384.

Kim, C. H. (2008) Regulation of FoxP3 regulatory T cells and Th17 cells by retinoids. *Clin Dev Immunol* 2008, 416910.

Kim, D. H., Lee, J. W., Kim, I. S., Choi, S. Y., Lim, Y. Y., Kim, H. M., Kim, B. J. and Kim, M. N. (2012) Successful Treatment of Alopecia Areata with Topical Calcipotriol. *Annals of Dermatology* 24 (3), 341-344.

Klingberg, F., Hinz, B. and White, E. S. (2013) The myofibroblast matrix: implications for tissue repair and fibrosis. *The Journal of pathology* 229 (2), 298-309.

Klopot, A., Hance, K. W., Peleg, S., Barsony, J. and Fleet, J. C. (2007) Nucleo-cytoplasmic Cycling of the Vitamin D Receptor in the Enterocyte-Like Cell Line, Caco-2. *Journal of cellular biochemistry* 100 (3), 617-628.

Kobayashi, T., Okumura, H., Hashimoto, K., Asada, H., Inui, S. and Yoshikawa, K. (1998) Synchronization of normal human keratinocyte in culture: its application to the analysis of 1,25-dihydroxyvitamin D3 effects on cell cycle. *J Dermatol Sci* 17 (2), 108-14.

Kohyama, T., Yamauchi, Y., Takizawa, H., Itakura, S., Kamitani, S., Kato, J. and Nagase, T. (2008) Clarithromycin inhibits fibroblast migration. *Respiratory Medicine* 102 (12), 1769-1776.

Kong, J., Li, X. J., Gavin, D., Jiang, Y. and Li, Y. C. (2002) Targeted expression of human vitamin d receptor in the skin promotes the initiation of the postnatal hair follicle cycle and rescues the alopecia in vitamin D receptor null mice. *J Invest Dermatol* 118 (4), 631-8.

Kong, J., Li, Y. C., Li, X. J., Gavin, D. and Jiang, Y. (2002) Targeted Expression of Human Vitamin D Receptor in the Skin Promotes the Initiation of the Postnatal Hair Follicle Cycle and Rescues the Alopecia in Vitamin D Receptor Null Mice. *Journal of Investigative Dermatology* 118 (4), 631-638.

Kongsbak, M., Levring, T. B., Geisler, C. and von Essen, M. R. (2013) The vitamin d receptor and T cell function. *Front Immunol* 4, 148.

Krafts, K. P. (2010) Tissue repair: The hidden drama. *Organogenesis* 6 (4), 225-233.

Krajisnik, T., Bjorklund, P., Marsell, R., Ljunggren, O., Akerstrom, G., Jonsson, K. B., Westin, G. and Larsson, T. E. (2007) Fibroblast growth factor-23 regulates parathyroid hormone and 1alpha-hydroxylase expression in cultured bovine parathyroid cells. *J Endocrinol* 195 (1), 125-31.

Krampert, M., Bloch, W., Sasaki, T., Bugnon, P., Rulicke, T., Wolf, E., Aumailley, M., Parks, W. C. and Werner, S. (2004) Activities of the matrix metalloproteinase stromelysin-2 (MMP-10) in matrix degradation and keratinocyte organization in wounded skin. *Mol Biol Cell* 15 (12), 5242-54.

Kratz, G. (1998) Modeling of wound healing processes in human skin using tissue culture. *Microsc Res Tech* 42 (5), 345-50.

Kuo, Y. T., Kuo, C. H., Lam, K. P., Chu, Y. T., Wang, W. L., Huang, C. H. and Hung, C. H. (2010) Effects of Vitamin D3 on Expression of Tumor Necrosis Factor-α and Chemokines by Monocytes. *Journal of food science* 75 (6).

Kyriakides, T. R., Wulsin, D., Skokos, E. A., Fleckman, P., Pirrone, A., Shipley, J. M., Senior, R. M. and Bornstein, P. (2009) Mice that lack matrix metalloproteinase-9 display delayed wound healing associated with delayed reepithelization and disordered collagen fibrillogenesis. *Matrix biology : journal of the International Society for Matrix Biology* 28 (2), 65-73.

Kyriakides, T. R., Wulsin, D., Skokos, E. A., Fleckman, P., Pirrone, A., Shipley, J. M., Senior, R. M. and Bornstein, P. (2009) Mice that lack matrix metalloproteinase-9 display delayed wound healing associated with delayed reepithelization and disordered collagen fibrillogenesis. *Matrix Biol* 28 (2), 65-73.

Lagishetty, V., Misharin, A. V., Liu, N. Q., Lisse, T. S., Chun, R. F., Ouyang, Y., McLachlan, S. M., Adams, J. S. and Hewison, M. (2010) Vitamin D deficiency in mice impairs colonic antibacterial activity and predisposes to colitis. *Endocrinology* 151 (6), 2423-32.

Lam, A., Flozak, A., Russell, S., Wei, J., Jain, M., M Mutlu, G., R Scott Budinger, G., Feghali-Bostwick, C., Varga, J. and Gottardi, C. (2011) *Nuclear β-Catenin Is Increased in Systemic Sclerosis Pulmonary Fibrosis and Promotes Lung Fibroblast Migration and Proliferation.* Vol. 45.

Lamouille, S., Xu, J. and Derynck, R. (2014) Molecular mechanisms of epithelial–mesenchymal transition. *Nature reviews. Molecular cell biology* 15 (3), 178-196.

Landén, N. X., Li, D. and Ståhle, M. (2016) Transition from inflammation to proliferation: a critical step during wound healing. *Cellular and Molecular Life Sciences* 73 (20), 3861-3885.

Larsson, D., Hagberg, M., Malek, N., Kjellberg, C., Senneberg, E., Tahmasebifar, N. and Johansson, V. (2008) Membrane initiated signaling by 1,25alpha-dihydroxyvitamin D3 in LNCaP prostate cancer cells. *Adv Exp Med Biol* 617, 573-9.

Lavi-Moshayoff, V., Wasserman, G., Meir, T., Silver, J. and Naveh-Many, T. (2010) PTH increases FGF23 gene expression and mediates the high-FGF23 levels of experimental kidney failure: a bone parathyroid feedback loop. *Am J Physiol Renal Physiol* 299 (4), F882-9.

Lechapt-Zalcman, E., Pruliere-Escabasse, V., Advenier, D., Galiacy, S., Charriere-Bertrand, C., Coste, A., Harf, A., d'Ortho, M. P. and Escudier, E. (2006) Transforming growth factor-beta1 increases airway wound repair via MMP-2 upregulation: a new pathway for epithelial wound repair? *Am J Physiol Lung Cell Mol Physiol* 290 (6), L1277-82.

Lee, J. and Youn, J. I. (1998) The photoprotective effect of 1,25-dihydroxyvitamin D3 on ultraviolet light B-induced damage in keratinocyte and its mechanism of action. *J Dermatol Sci* 18 (1), 11-8.

Lee, S. A., Yang, H. W., Um, J. Y., Shin, J. M., Park, I. H. and Lee, H. M. (2017) Vitamin D attenuates myofibroblast differentiation and extracellular matrix accumulation in nasal polyp-derived fibroblasts through smad2/3 signaling pathway. *Sci Rep* 7 (1), 7299.

Levayer, R. and Lecuit, T. (2008) Breaking down EMT. *Nat Cell Biol* 10 (7), 757-9.

Levy, V., Lindon, C., Zheng, Y., Harfe, B. D. and Morgan, B. A. (2007) Epidermal stem cells arise from the hair follicle after wounding. *Faseb j* 21 (7), 1358-66.

Li, J., Zhang, Y. P. and Kirsner, R. S. (2003) Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix. *Microsc Res Tech* 60 (1), 107-14.

Li, M., Sun, X., Ma, L., Jin, L., Zhang, W., Xiao, M. and Yu, Q. (2017) *SDF-1/CXCR4 axis induces human dental pulp stem cell migration through FAK/PI3K/Akt and GSK3β/β-catenin pathways.* Vol. 7.

Li, S., Niu, G., Wu, Y., Du, G., Huang, C., Yin, X., Liu, Z., Song, C. and Leng, H. (2016) Vitamin D prevents articular cartilage erosion by regulating collagen II turnover through TGF-β1 in ovariectomized rats. *Osteoarthritis and Cartilage* 24 (2), 345-353.

Li, W., Fan, J., Chen, M., Guan, S., Sawcer, D., Bokoch, G. M. and Woodley, D. T. (2004) Mechanism of Human Dermal Fibroblast Migration Driven by Type I Collagen and Platelet-derived Growth Factor-BB. *Molecular Biology of the Cell* 15 (1), 294-309.

Li, Y., Spataro, B. C., Yang, J., Dai, C. and Liu, Y. (2005) 1,25-dihydroxyvitamin D inhibits renal interstitial myofibroblast activation by inducing hepatocyte growth factor expression. *Kidney Int* 68 (4), 1500-10.

Lindh, J. D., Bjorkhem-Bergman, L. and Eliasson, E. (2012) Vitamin D and drug-metabolising enzymes. *Photochemical & Photobiological Sciences* 11 (12), 1797-1801.

Lindner, D., Zietsch, C., Becher, P. M., Schulze, K., Schultheiss, H.-P., Tsch, #xf6, pe, C. and Westermann, D. (2012) Differential Expression of Matrix Metalloproteases in Human Fibroblasts with Different Origins. *Biochemistry Research International* 2012, 10.

Lindner, D., Zietsch, C., Becher, P. M., Schulze, K., Schultheiss, H.-P., Tschöpe, C. and Westermann, D. (2012) Differential expression of matrix metalloproteases in human fibroblasts with different origins. *Biochemistry research international* 2012.

Lips, P. (2007) Relative value of 25 (OH) D and 1, 25 (OH) 2D measurements. *Journal of Bone and Mineral Research* 22 (11), 1668-1671.

Liu, Y., Beyer, A. and Aebersold, R. (2016) On the Dependency of Cellular Protein Levels on mRNA Abundance. *Cell* 165 (3), 535-50.

Lodish H, B. A., Zipursky SL, et al. (2000) Collagen: The Fibrous Proteins of the Matrix. *Molecular Cell Biology.* 4th edition edition. New York: W.H. Freeman.

Lou, Y. R., Molnar, F., Perakyla, M., Qiao, S., Kalueff, A. V., St-Arnaud, R., Carlberg, C. and Tuohimaa, P. (2010) 25-Hydroxyvitamin D(3) is an agonistic vitamin D receptor ligand. *J Steroid Biochem Mol Biol* 118 (3), 162-70.

Lovell, C., Smolenski, K., Duance, V., Light, N., Young, S. and Dyson, M. (1987) Type I and III collagen content and fibre distribution in normal human skin during ageing. *British Journal of Dermatology* 117 (4), 419-428.

Lu, X., Chen, Z., Mylarapu, N. and Watsky, M. A. (2017) Effects of 1,25 and 24,25 Vitamin D on Corneal Epithelial Proliferation, Migration and Vitamin D Metabolizing and Catabolizing Enzymes. *Scientific Reports* 7, 16951.

Lu, Y., Papagerakis, P., Yamakoshi, Y., Hu, J. C., Bartlett, J. D. and Simmer, J. P. (2008) Functions of KLK4 and MMP-20 in dental enamel formation. *Biol Chem* 389 (6), 695-700.

Lu, Z., Chen, T. C., Zhang, A., Persons, K. S., Kohn, N., Berkowitz, R., Martinello, S. and Holick, M. F. (2007) An evaluation of the vitamin D-3 content in fish: Is the vitamin D content adequate to satisfy the dietary requirement for vitamin D? *Journal of Steroid Biochemistry and Molecular Biology* 103 (3-5), 642-644.

Luderer, H. F., Nazarian, R. M., Zhu, E. D. and Demay, M. B. (2013) Ligand-Dependent Actions of the Vitamin D Receptor Are Required for Activation of TGF-β Signaling during the Inflammatory Response to Cutaneous Injury. *Endocrinology* 154 (1), 16-24.

Lulevich, V., Yang, H.-y., Isseroff, R. R. and Liu, G.-y. (2010) Single cell mechanics of keratinocyte cells. *Ultramicroscopy* 110 (12), 1435-1442.

Lynch, S. E., Nixon, J. C., Colvin, R. B. and Antoniades, H. N. (1987) Role of platelet-derived growth factor in wound healing: synergistic effects with other growth factors. *Proceedings of the National Academy of Sciences of the United States of America* 84 (21), 7696-7700.

MacLaughlin, J. and Holick, M. F. (1985) Aging decreases the capacity of human skin to produce vitamin D3. *Journal of Clinical Investigation* 76 (4), 1536-1538.

MacLaughlin, J. A., Gange, W., Taylor, D., Smith, E. and Holick, M. F. (1985) Cultured psoriatic fibroblasts from involved and uninvolved sites have a partial but not absolute resistance to the proliferation-inhibition activity of 1,25-dihydroxyvitamin D3. *Proceedings of the National Academy of Sciences of the United States of America* 82 (16), 5409-5412.

MacNeil, S. (2007) Progress and opportunities for tissue-engineered skin. *Nature* 445 (7130), 874-880.

Maier, T., Güell, M. and Serrano, L. (2009) Correlation of mRNA and protein in complex biological samples. *FEBS Letters* 583 (24), 3966-3973.

Maione, A. G., Brudno, Y., Stojadinovic, O., Park, L. K., Smith, A., Tellechea, A., Leal, E. C., Kearney, C. J., Veves, A., Tomic-Canic, M., Mooney, D. J. and Garlick, J. A. (2015) Three-Dimensional Human Tissue Models That Incorporate Diabetic Foot Ulcer-Derived Fibroblasts Mimic In Vivo Features of Chronic Wounds. *Tissue Engineering. Part C, Methods* 21 (5), 499-508.

Mann, A., Breuhahn, K., Schirmacher, P. and Blessing, M. (2001) Keratinocyte-derived granulocyte-macrophage colony stimulating factor accelerates wound healing: Stimulation of keratinocyte proliferation, granulation tissue formation, and vascularization. *J Invest Dermatol* 117 (6), 1382-90.

Marangoni, R. G., Korman, B. D., Wei, J., Wood, T. A., Graham, L. V., Whitfield, M. L., Scherer, P. E., Tourtellotte, W. G. and Varga, J. (2015) Myofibroblasts in murine cutaneous fibrosis originate from adiponectin-positive intradermal progenitors. *Arthritis Rheumatol* 67 (4), 1062-73.

Marionnet, C., Pierrard, C., Vioux-Chagnoleau, C., Sok, J., Asselineau, D. and Bernerd, F. (2006) Interactions between Fibroblasts and Keratinocytes in Morphogenesis of Dermal Epidermal Junction in a Model of Reconstructed Skin. *Journal of Investigative Dermatology* 126 (5), 971-979.

Martin, P. (1997) Wound healing--aiming for perfect skin regeneration. *Science* 276 (5309), 75-81.

Mason (2000) *Vitamin D Endocrine System: Structural, Biological, Genetic and Clinical Aspects.* Riverside: University of California.

Masuyama, H., Brownfield, C. M., St-Arnaud, R. and MacDonald, P. N. (1997) Evidence for Ligand-Dependent Intramolecular Folding of the AF-2 Domain in Vitamin D Receptor-Activated Transcription and Coactivator Interaction. *Molecular Endocrinology* 11 (10), 1507-1517.

Matsuda, S. and Kitagishi, Y. (2013) Peroxisome Proliferator-Activated Receptor and Vitamin D Receptor Signaling Pathways in Cancer Cells. *Cancers* 5 (4), 1261-1270.

McCollum, E. V., Simmonds, N., Becker, J. E. and Shipley, P. (1922) Studies on experimental rickets XXI. An experimental demonstration of the existence of a vitamin which promotes calcium deposition. *Journal of Biological Chemistry* 53 (2), 293-312.

McDonald, P. P. (2004) Transcriptional Regulation in Neutrophils: Teaching Old Cells New Tricks. *Advances in Immunology.* Vol. 82. Academic Press. 1-48.

McElwee, K. J., Kissling, S., Wenzel, E., Huth, A. and Hoffmann, R. (2003) Cultured peribulbar dermal sheath cells can induce hair follicle development and contribute to the dermal sheath and dermal papilla. *J Invest Dermatol* 121 (6), 1267-75.

McGuire, J. K., Li, Q. and Parks, W. C. (2003) Matrilysin (matrix metalloproteinase-7) mediates E-cadherin ectodomain shedding in injured lung epithelium. *Am J Pathol* 162 (6), 1831-43.

Meigel, W. N., Gay, S. and Weber, L. (1977) Dermal architecture and collagen type distribution. *Arch Dermatol Res* 259 (1), 1-10.

Meindl, S., Rot, A., Hoetzenecker, W., Kato, S., Cross, H. S. and Elbe-Burger, A. (2005) Vitamin D receptor ablation alters skin architecture and homeostasis of dendritic epidermal T cells. *Br J Dermatol* 152 (2), 231-41.

Meredith, A., Boroomand, S., Carthy, J., Luo, Z. and McManus, B. (2015) 1, 25 Dihydroxyvitamin D3 inhibits TGFβ1-mediated primary human cardiac myofibroblast activation. *PloS one* 10 (6), e0128655.

Merkel, J. R., DiPaolo, B. R., Hallock, G. G. and Rice, D. C. (1988) Type I and type III collagen content of healing wounds in fetal and adult rats. *Proc Soc Exp Biol Med* 187 (4), 493-7.

Meyer, M. B., Zella, L. A., Nerenz, R. D. and Pike, J. W. (2007) Characterizing early events associated with the activation of target genes by 1, 25-dihydroxyvitamin D3 in mouse kidney and intestine in vivo. *Journal of Biological Chemistry* 282 (31), 22344-22352.

Micallef, L., Vedrenne, N., Billet, F., Coulomb, B., Darby, I. A. and Desmouliere, A. (2012) The myofibroblast, multiple origins for major roles in normal and pathological tissue repair. *Fibrogenesis Tissue Repair* 5 (Suppl 1), S5.

Milde, P., Hauser, U., Simon, T., Mall, G., Ernst, V., Haussler, M. R., Frosch, P. and Rauterberg, E. W. (1991) Expression of 1, 25-dihydroxyvitamin D 3 receptors in normal and psoriatic skin. *Journal of investigative dermatology* 97 (2), 230-239.

Mirastschijski, U., Zhou, Z., Rollman, O., Tryggvason, K. and Agren, M. S. (2004) Wound healing in membrane-type-1 matrix metalloproteinase-deficient mice. *J Invest Dermatol* 123 (3), 600-2.

Mistriotis, P. and Andreadis, S. T. (2013) Hair Follicle: A Novel Source of Multipotent Stem Cells for Tissue Engineering and Regenerative Medicine. *Tissue Engineering. Part B, Reviews* 19 (4), 265-278.

Mitchison, T. J. and Cramer, L. P. (1996) Actin-based cell motility and cell locomotion. *Cell* 84 (3), 371-379.

Mohan, R., Chintala, S. K., Jung, J. C., Villar, W. V. L., McCabe, F., Russo, L. A., Lee, Y., McCarthy, B. E., Wollenberg, K. R., Jester, J. V., Wang, M., Welgus, H. G., Shipley, J. M., Senior, R. M. and Fini, M. E. (2002) Matrix Metalloproteinase Gelatinase B (MMP-9) Coordinates and Effects Epithelial Regeneration. *Journal of Biological Chemistry* 277 (3), 2065-2072.

Mohr, S., Garland, C., Gorham, E. and Garland, F. (2008) The association between ultraviolet B irradiance, vitamin D status and incidence rates of type 1 diabetes in 51 regions worldwide. *Diabetologia* 51 (8), 1391-1398.

Moll, I., Roessler, M., Brandner, J. M., Eispert, A. C., Houdek, P. and Moll, R. (2005) Human Merkel cells--aspects of cell biology, distribution and functions. *Eur J Cell Biol* 84 (2-3), 259-71.

Molnar, J. A., Underdown, M. J. and Clark, W. A. (2014) Nutrition and chronic wounds. *Advances in wound care* 3 (11), 663-681.

Moore, J. T., Collins, J. L. and Pearce, K. H. (2006) The nuclear receptor superfamily and drug discovery. *ChemMedChem* 1 (5), 504-23.

Morelli, S., de Boland, A. R. and Boland, R. L. (1993) Generation of inositol phosphates, diacylglycerol and calcium fluxes in myoblasts treated with 1,25-dihydroxyvitamin D3. *Biochem J* 289 (Pt 3), 675-9.

Morrill, G. A., Kostellow, A. B. and Gupta, R. K. (2016) The role of receptor topology in the vitamin D3 uptake and Ca2+ response systems. *Biochemical and Biophysical Research Communications* 477 (4), 834-840.

Mostafa, W. Z. and Hegazy, R. A. (2015) Vitamin D and the skin: Focus on a complex relationship: A review. *Journal of Advanced Research* 6 (6), 793-804.

Moulin, V. (1995) Growth factors in skin wound healing. *Eur J Cell Biol* 68 (1), 1-7.

Mpandzou, G., Ait Ben Haddou, E., Regragui, W., Benomar, A. and Yahyaoui, M. (2016) Vitamin D deficiency and its role in neurological conditions: A review. *Revue neurologique* 172 (2), 109-22.

Müller, M. J. and Volmer, D. A. (2015) Mass Spectrometric Profiling of Vitamin D Metabolites beyond 25-Hydroxyvitamin D. *Clinical Chemistry* 61 (8), 1033-1048.

Munetsuna, E., Kawanami, R., Nishikawa, M., Ikeda, S., Nakabayashi, S., Yasuda, K., Ohta, M., Kamakura, M., Ikushiro, S. and Sakaki, T. (2014) Anti-proliferative activity of 25-hydroxyvitamin D3 in human prostate cells. *Molecular and Cellular Endocrinology* 382 (2), 960-970.

Murphy, G. and Nagase, H. (2008) Progress in matrix metalloproteinase research. *Molecular aspects of medicine* 29 (5), 290-308.

Nagase, H. and Woessner, J. F. (1999) Matrix metalloproteinases. *Journal of Biological chemistry* 274 (31), 21491-21494.

Nagavarapu, U., Relloma, K. and Herron, G. S. (2002) Membrane type 1 matrix metalloproteinase regulates cellular invasiveness and survival in cutaneous epidermal cells. *J Invest Dermatol* 118 (4), 573-81.

Natarajan, V. T., Ganju, P., Ramkumar, A., Grover, R. and Gokhale, R. S. (2014) Multifaceted pathways protect human skin from UV radiation. *Nat Chem Biol* 10 (7), 542-551.

Nayak, B. K. and Hazra, A. (2011) How to choose the right statistical test? *Indian Journal of Ophthalmology* 59 (2), 85-86.

Nebbioso, M., Buomprisco, G., Pascarella, A. and Pescosolido, N. (2015) Modulatory Effects of 1,25-dihydroxyvitamin D3 on Eye Disorders: A Critical Review. *Critical Reviews in Food Science and Nutrition*, 00-00.

Nemere, I., Farach-Carson, M., Rohe, B., Sterling, T., Norman, A., Boyan, B. and Safford, S. (2004) Ribozyme knockdown functionally links a 1, 25 (OH) 2D3 membrane binding protein (1, 25D3-MARRS) and phosphate uptake in intestinal cells. *Proceedings of the national academy of sciences of the United States of America* 101 (19), 7392-7397.

Nemere, I., Garbi, N., Hammerling, G. J. and Khanal, R. C. (2010) Intestinal cell calcium uptake and the targeted knockout of the 1,25D3-MARRS (membrane-associated, rapid response steroid-binding) receptor/PDIA3/Erp57. *J Biol Chem* 285 (41), 31859-66.

Neub, A., Houdek, P., Ohnemus, U., Moll, I. and Brandner, J. M. (2007) Biphasic Regulation of AP-1 Subunits during Human Epidermal Wound Healing. *Journal of Investigative Dermatology* 127 (10), 2453-2462.

Niyonsaba, F., Nagaoka, I. and Ogawa, H. (2006) Human defensins and cathelicidins in the skin: beyond direct antimicrobial properties. *Crit Rev Immunol* 26 (6), 545-76.

Norlin, M., Lundqvist, J., Ellfolk, M., Hellstrom Pigg, M., Gustafsson, J. and Wikvall, K. (2017) Drug-Mediated Gene Regulation of Vitamin D3 Metabolism in Primary Human Dermal Fibroblasts. *Basic Clin Pharmacol Toxicol* 120 (1), 59-63.

Norlin, M., Lundqvist, J., Ellfolk, M., Hellström Pigg, M., Gustafsson, J. and Wikvall, K. (2017) Drug-Mediated Gene Regulation of Vitamin D3 Metabolism in Primary Human Dermal Fibroblasts. *Basic & clinical pharmacology & toxicology* 120 (1), 59-63.

Norman, A. W. (2006) Minireview: vitamin D receptor: new assignments for an already busy receptor. *Endocrinology* 147 (12), 5542-8.

Norman, A. W. (2008) From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *The American journal of clinical nutrition* 88 (2), 491S-499S.

Norman, A. W. (2011) *Chemistry of Vitamin D.* 2011. http://vitamind.ucr.edu/about/: *http://vitamind.ucr.edu/about/*

Norris, D. A. (2005) Mechanisms of action of topical therapies and the rationale for combination therapy. *J Am Acad Dermatol* 53 (1 Suppl 1), S17-25.

Nurnberg, B., Graber, S., Gartner, B., Geisel, J., Pfohler, C., Schadendorf, D., Tilgen, W. and Reichrath, J. (2009) Reduced serum 25-hydroxyvitamin D levels in stage IV melanoma patients. *Anticancer Res* 29 (9), 3669-74.

Nwomeh, B. C., Liang, H. X., Cohen, I. K. and Yager, D. R. (1999) MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers. *J Surg Res* 81 (2), 189-95.

Nwomeh, B. C., Liang, H. X., Diegelmann, R. F., Cohen, I. K. and Yager, D. R. (1998) Dynamics of the matrix metalloproteinases MMP-1 and MMP-8 in acute open human dermal wounds. *Wound Repair Regen* 6 (2), 127-34.

Nykjaer, A., Dragun, D., Walther, D., Vorum, H., Jacobsen, C., Herz, J., Melsen, F., Christensen, E. I. and Willnow, T. E. (1999) An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell* 96 (4), 507-515.

O'Toole, E. A., Palicharla, P., West, D. P., Woodley, D. T. and Nasca, M. R. (1999) Thalidomide Increases Human Keratinocyte Migration and Proliferation. *Journal of Investigative Dermatology* 113 (5), 720-724.

Oda, Y., Tu, C. L., Menendez, A., Nguyen, T. and Bikle, D. D. (2015) Vitamin D and calcium regulation of epidermal wound healing. *J Steroid Biochem Mol Biol*.

Ohyama, Y. and Shinki, T. (2016) Cholecalciferol. *Handbook of Hormones. Comparative Endocrinology for Basic and Clinical Research.*

Ohyama, Y. and Shinki, T. (2016) Subchapter 97B - Cholecalciferol A2 - Takei, Yoshio. In Ando, H. and Tsutsui, K. (editors) *Handbook of Hormones.* San Diego: Academic Press. 551-e97B-3.

Oikawa, A. and Nakayasu, M. (1974) Stimulation of melanogenesis in cultured melanoma cells by calciferols. *FEBS letters* 42 (1), 32-35.

Oliveri, B., Mastaglia, S. R., Brito, G. M., Seijo, M., Keller, G. A., Somoza, J., Diez, R. A. and Di Girolamo, G. (2015) Vitamin D3 seems more appropriate than D2 to sustain adequate levels of 25OHD: a pharmacokinetic approach. *European Journal of Clinical Nutrition* 69 (6), 697-702.

Orchinik, M., Hastings, N., Witt, D. and McEwen, B. S. (1997) High-affinity binding of corticosterone to mammalian neuronal membranes: possible role of corticosteroid binding globulin. *J Steroid Biochem Mol Biol* 60 (3-4), 229-36.

Otte, J.-M., Zdebik, A.-E., Brand, S., Chromik, A. M., Strauss, S., Schmitz, F., Steinstraesser, L. and Schmidt, W. E. (2009) Effects of the cathelicidin LL-37 on intestinal epithelial barrier integrity. *Regulatory Peptides* 156 (1), 104-117.

Park, K. (2015) Role of Micronutrients in Skin Health and Function. *Biomolecules & Therapeutics* 23 (3), 207-217.

Pellicena, I. C. (2017) *The Role of Photoreceptors in Human Skin Physiology: Potential Targets For Light-Based Wound Healing Therapies*. PhD. University of Bradford.

Peng, X., Hawthorne, M., Vaishnav, A., St-Arnaud, R. and Mehta, R. G. (2009) 25-Hydroxyvitamin D3 is a natural chemopreventive agent against carcinogen induced precancerous lesions in mouse mammary gland organ culture. *Breast Cancer Res Treat* 113 (1), 31-41.

Pike, J. W. and Meyer, M. B. (2010) The Vitamin D Receptor: New Paradigms for the Regulation of Gene Expression by 1,25-Dihydroxyvitamin D(3). *Endocrinology and metabolism clinics of North America* 39 (2), 255-269.

Pilcher, B. K., Dumin, J. A., Sudbeck, B. D., Krane, S. M., Welgus, H. G. and Parks, W. C. (1997) The activity of collagenase-1 is required for

keratinocyte migration on a type I collagen matrix. *J Cell Biol* 137 (6), 1445-57.

Pillai, S., Bikle, D. D., Su, M. J., Ratnam, A. and Abe, J. (1995) 1,25-Dihydroxyvitamin D3 upregulates the phosphatidylinositol signaling pathway in human keratinocytes by increasing phospholipase C levels. *Journal of Clinical Investigation* 96 (1), 602-609.

Plikus, M. V., Guerrero-Juarez, C. F., Ito, M., Li, Y. R., Dedhia, P. H., Zheng, Y., Shao, M., Gay, D. L., Ramos, R., His, T.-C., Oh, J. W., Wang, X., Ramirez, A., Konopelski, S. E., Elzein, A., Wang, A., Supapannachart, R. J., Lee, H.-L., Lim, C. H., Nace, A., Guo, A., Treffeisen, E., Andl, T., Ramirez, R. N., Murad, R., Offermanns, S., Metzger, D., Chambon, P., Widgerow, A. D., Tuan, T.-L., Mortazavi, A., Gupta, R. K., Hamilton, B. A., Millar, S. E., Seale, P., Pear, W. S., Lazar, M. A. and Cotsarelis, G. (2017) Regeneration of fat cells from myofibroblasts during wound healing. *Science*.

Pols, H. A. P., Birkenhäger, J. C., Foekens, J. A. and van Leeuwen, J. P. T. M. (1990) Vitamin D: A modulator of cell proliferation and differentiation. *The Journal of Steroid Biochemistry and Molecular Biology* 37 (6), 873-876.

Pomari, E., Dalla Valle, L., Pertile, P., Colombo, L. and Thornton, M. J. (2014) Intracrine sex steroid synthesis and signaling in human epidermal keratinocytes and dermal fibroblasts. *The FASEB Journal*, fj. 14-251363.

Porter, A. M. W. (2001) Why do we have apocrine and sebaceous glands? *Journal of the Royal Society of Medicine* 94 (5), 236-237.

Praeger, F. C. and Gilchrest, B. A. (1986) Influence of increased extracellular calcium concentration and donor age on density-dependent growth inhibition of human fibroblasts. *Proc Soc Exp Biol Med* 182 (3), 315-21.

Pratt, A. J. and MacRae, I. J. (2009) The RNA-induced Silencing Complex: A Versatile Gene-silencing Machine. *The Journal of Biological Chemistry* 284 (27), 17897-17901.

Prosser, D. E. and Jones, G. (2004) Enzymes involved in the activation and inactivation of vitamin D. *Trends in Biochemical Sciences* 29 (12), 664-673.

Prufer, K. and Barsony, J. (2002) Retinoid X receptor dominates the nuclear import and export of the unliganded vitamin D receptor. *Mol Endocrinol* 16 (8), 1738-51.

Prüfer, K. and Barsony, J. (2002) Retinoid X receptor dominates the nuclear import and export of the unliganded vitamin D receptor. *Molecular Endocrinology* 16 (8), 1738-1751.

Prüfer, K., Racz, A., Lin, G. C. and Barsony, J. (2000) Dimerization with retinoid X receptors promotes nuclear localization and subnuclear targeting of vitamin D receptors. *Journal of Biological Chemistry* 275 (52), 41114-41123.

Prüfer, K., Veenstra, T. D., Jirikowski, G. F. and Kumar, R. (1999) Distribution of 1,25-dihydroxyvitamin D3 receptor immunoreactivity in the rat brain and spinal cord. *Journal of Chemical Neuroanatomy* 16 (2), 135-145.

Qi, X., Pramanik, R., Wang, J., Schultz, R. M., Maitra, R. K., Han, J., DeLuca, H. F. and Chen, G. (2002) The p38 and JNK Pathways Cooperate to

trans-Activate Vitamin D Receptor via c-Jun/AP-1 and Sensitize Human Breast Cancer Cells to Vitamin D3-induced Growth Inhibition. *Journal of Biological Chemistry* 277 (29), 25884-25892.

Quarles, L. D. (2012) Role of FGF23 in vitamin D and phosphate metabolism: implications in chronic kidney disease. *Exp Cell Res* 318 (9), 1040-8.

R Merkel, J., R DiPaolo, B., G Hallock, G. and C Rice, D. (1988) *Type I and Type III Collagen Content of Healing Wounds in Fetal and Adult Rats.* Vol. 187.

Racz, A. and Barsony, J. (1999) Hormone-dependent translocation of vitamin D receptors is linked to transactivation. *J Biol Chem* 274 (27), 19352-60.

Rambukkana, A., Bos, J. D., Irik, D., Menko, W. J., Kapsenberg, M. L. and Das, P. K. (1995) In situ behavior of human Langerhans cells in skin organ culture. *Lab Invest* 73 (4), 521-31.

Randall, V. A., Sundberg, J. P. and Philpott, M. P. (2003) Animal and in vitro Models for the Study of Hair Follicles. *J Investig Dermatol Symp Proc* 8 (1), 39-45.

Razzaghi, R., Pourbagheri, H., Momen-Heravi, M., Bahmani, F., Shadi, J., Soleimani, Z. and Asemi, Z. (2017) The effects of vitamin D supplementation on wound healing and metabolic status in patients with diabetic foot ulcer: A randomized, double-blind, placebo-controlled trial. *J Diabetes Complications* 31 (4), 766-772.

Rebsamen, M. C., Sun, J., Norman, A. W. and Liao, J. K. (2002) 1alpha,25-dihydroxyvitamin D3 induces vascular smooth muscle cell migration via activation of phosphatidylinositol 3-kinase. *Circ Res* 91 (1), 17-24.

Redfern, C. P. and Todd, C. (1992) Retinoic acid receptor expression in human skin keratinocytes and dermal fibroblasts in vitro. *J Cell Sci* 102 (Pt 1), 113-21.

Reichrath, J., Schilli, M., Kerber, A., Bahmer, F. A., Czarnetzki, B. M. and Paus, R. (1994) Hair follicle expression of 1,25-dihydroxyvitamin D3 receptors during the murine hair cycle. *Br J Dermatol* 131 (4), 477-82.

Reichrath, J., Schilli, M., Kerber, A., Bahmer, F. A., Czarnetzki, B. M. and Paus, R. (1994) Hair follicle expression of 1,25-dihydroxyvitamin D3 receptors during the murine hair cycle. *British Journal of Dermatology* 131 (4), 477-482.

Reinke, J. M. and Sorg, H. (2012) Wound Repair and Regeneration. *European Surgical Research* 49 (1), 35-43.

Reiske, H. R., Kao, S.-C., Cary, L. A., Guan, J.-L., Lai, J.-F. and Chen, H.-C. (1999) Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. *Journal of Biological Chemistry* 274 (18), 12361-12366.

Reynolds, A. J., Lawrence, C., Cserhalmi-Friedman, P. B., Christiano, A. M. and Jahoda, C. A. (1999) Trans-gender induction of hair follicles. *Nature* 402 (6757), 33-4.

Richard, C. L., Farach-Carson, M. C., Rohe, B., Nemere, I. and Meckling, K. A. (2010) Involvement of 1,25D3-MARRS (membrane associated, rapid response steroid-binding), a novel vitamin D receptor, in growth inhibition of breast cancer cells. *Exp Cell Res* 316 (5), 695-703.

Risinger, G. M., Updike, D. L., Bullen, E. C., Tomasek, J. J. and Howard, E. W. (2010) TGF-β suppresses the upregulation of MMP-2 by vascular smooth muscle cells in response to PDGF-BB. *American Journal of Physiology - Cell Physiology* 298 (1), C191-C201.

Ritter, C. S., Armbrecht, H. J., Slatopolsky, E. and Brown, A. J. (2006) 25-Hydroxyvitamin D3 suppresses PTH synthesis and secretion by bovine parathyroid cells. *Kidney International* 70 (4), 654-659.

Rittié, L., Sachs, D. L., Orringer, J. S., Voorhees, J. J. and Fisher, G. J. (2013) Eccrine Sweat Glands are Major Contributors to Reepithelialization of Human Wounds. *The American Journal of Pathology* 182 (1), 163-171.

Robins, S. P., Milne, G., Duncan, A., Davies, C., Butt, R., Greiling, D. and James, I. T. (2003) Increased Skin Collagen Extractability and Proportions of Collagen Type III Are Not Normalized after 6 Months Healing of Human Excisional Wounds. *Journal of Investigative Dermatology* 121 (2), 267-272.

Roh, S.-S., Lee, M.-H., Hwang, Y.-L., Song, H.-H., Jin, M. H., Park, S. G., Lee, C. K., Kim, C. D., Yoon, T.-J. and Lee, J.-H. (2010) Stimulation of the Extracellular Matrix Production in Dermal Fibroblasts by Velvet Antler Extract. *Annals of Dermatology* 22 (2), 173-179.

Rohani, M. G. and Parks, W. C. (2015) Matrix remodeling by MMPs during wound repair. *Matrix Biology* 44-46, 113-121.

Rohe, B., Safford, S. E., Nemere, I. and Farach-Carson, M. C. (2005) Identification and characterization of 1,25D3-membrane-associated rapid response, steroid (1,25D3-MARRS)-binding protein in rat IEC-6 cells. *Steroids* 70 (5-7), 458-63.

Rosen, H., Reshef, A., Maeda, N., Lippoldt, A., Shpizen, S., Triger, L., Eggertsen, G., Bjorkhem, I. and Leitersdorf, E. (1998) Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. *J Biol Chem* 273 (24), 14805-12.

Ross AC, T. C., Yaktine AL (2011) *Dietary Reference Intakes for Calcium and Vitamin D.* Washington (DC): National Academies Press (US). http://www.ncbi.nlm.nih.gov/books/NBK56061/ Accessed 30 March.

Rothan, H. A., Djordjevic, I., Bahrani, H., Paydar, M., Ibrahim, F., Rahmanh, N. A. and Yusof, R. (2014) Three-Dimensional Culture Environment Increases the Efficacy of Platelet Rich Plasma Releasate in Prompting Skin Fibroblast Differentiation and Extracellular Matrix Formation. *International Journal of Medical Sciences* 11 (10), 1029-1038.

Rotty, J. D. and Coulombe, P. A. (2012) A wound-induced keratin inhibits Src activity during keratinocyte migration and tissue repair. *The Journal of Cell Biology* 197 (3), 381-389.

Row, Paula E., Clague, Michael J. and Urbé, S. (2005) Growth factors induce differential phosphorylation profiles of the Hrs–STAM complex: a common node in signalling networks with signal-specific properties. *Biochemical Journal* 389 (Pt 3), 629-636.

Rubin, J. S., Bottaro, D. P., Chedid, M., Miki, T., Ron, D., Cheon, G., Taylor, W. G., Fortney, E., Sakata, H., Finch, P. W. and et al. (1995) Keratinocyte growth factor. *Cell Biol Int* 19 (5), 399-411.

Ruth Wu-Wong, J., Nakane, M., Ma, J., Dixon, D. and Gagne, G. (2006) Vitamin D receptor (VDR) localization in human promyelocytic leukemia cells. *Leukemia & lymphoma* 47 (4), 727-732.

Sakai, Y. and Demay, M. B. (2000) Evaluation of keratinocyte proliferation and differentiation in vitamin D receptor knockout mice. *Endocrinology* 141 (6), 2043-9.

Sakai, Y., Kishimoto, J. and Demay, M. B. (2001) Metabolic and cellular analysis of alopecia in vitamin D receptor knockout mice. *J Clin Invest* 107 (8), 961-6.

Sanchez-Martinez, R., Zambrano, A., Castillo, A. I. and Aranda, A. (2008) Vitamin D-dependent recruitment of corepressors to vitamin D/retinoid X receptor heterodimers. *Mol Cell Biol* 28 (11), 3817-29.

Sánchez-Martínez, R., Zambrano, A., Castillo, A. I. and Aranda, A. (2008) Vitamin D-Dependent Recruitment of Corepressors to Vitamin D/Retinoid X Receptor Heterodimers. *Molecular and Cellular Biology* 28 (11), 3817-3829.

Sanger, J. M., Dabiri, G., Mittal, B., Kowalski, M. A., Haddad, J. G. and Sanger, J. W. (1990) Disruption of microfilament organization in living nonmuscle cells by microinjection of plasma vitamin D-binding protein or DNase I. *Proc Natl Acad Sci U S A* 87 (14), 5474-8.

Sato, T., Kirimura, Y. and Mori, Y. (1997) The co-culture of dermal fibroblasts with human epidermal keratinocytes induces increased prostaglandin E2 production and cyclooxygenase 2 activity in fibroblasts. *J Invest Dermatol* 109 (3), 334-9.

Schauber, J., Dorschner, R. A., Coda, A. B., Buchau, A. S., Liu, P. T., Kiken, D., Helfrich, Y. R., Kang, S., Elalieh, H. Z., Steinmeyer, A., Zugel, U., Bikle, D. D., Modlin, R. L. and Gallo, R. L. (2007) Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J Clin Invest* 117 (3), 803-11.

Schneider, M. R., Schmidt-Ullrich, R. and Paus, R. (2009) The Hair Follicle as a Dynamic Miniorgan. *Current Biology* 19 (3), R132-R142.

Schräder, M., Müller, K. M. and Carlberg, C. (1994) Specificity and flexibility of vitamin D signaling. Modulation of the activation of natural vitamin D response elements by thyroid hormone. *Journal of Biological Chemistry* 269 (8), 5501-5504.

Schroder, J. M. (1995) Cytokine networks in the skin. *J Invest Dermatol* 105 (1 Suppl), 20s-24s.

Schuessler, M., Astecker, N., Herzig, G., Vorisek, G. and Schuster, I. (2001) Skin is an autonomous organ in synthesis, two-step activation and degradation of vitamin D(3): CYP27 in epidermis completes the set of essential vitamin D(3)-hydroxylases. *Steroids* 66 (3-5), 399-408.

Schuster, I., Egger, H., Astecker, N., Herzig, G., Schussler, M. and Vorisek, G. (2001) Selective inhibitors of CYP24: mechanistic tools to explore vitamin D metabolism in human keratinocytes. *Steroids* 66 (3-5), 451-62.

Schwalfenberg, G. K. (2011) A review of the critical role of vitamin D in the functioning of the immune system and the clinical implications of vitamin D deficiency. *Mol Nutr Food Res* 55 (1), 96-108.

Schwartz, Z., Ehland, H., Sylvia, V. L., Larsson, D., Hardin, R. R., Bingham, V., Lopez, D., Dean, D. D. and Boyan, B. D. (2002) 1α,25-Dihydroxyvitamin D3 and 24R,25-Dihydroxyvitamin D3 Modulate Growth Plate Chondrocyte Physiology via Protein Kinase C-Dependent Phosphorylation of Extracellular Signal-Regulated Kinase 1/2 Mitogen-Activated Protein Kinase. *Endocrinology* 143 (7), 2775-2786.

Seomun, Y., Kim, J. T. and Joo, C. K. (2008) MMP-14 mediated MMP-9 expression is involved in TGF-beta1-induced keratinocyte migration. *J Cell Biochem* 104 (3), 934-41.

Seyhan, H., Stromps, J.-P., Demir, E., Fuchs, P. C. and Kopp, J. (2018) Vitamin D deficiency may stimulate fibroblasts in Dupuytreńs disease via mitochondrial increased reactive oxygen species through upregulating transforming growth factor-β1. *Medical Hypotheses*.

Shaffer, P. L. and Gewirth, D. T. (2002) Structural basis of VDR–DNA interactions on direct repeat response elements. *The EMBO Journal* 21 (9), 2242-2252.

Shaw, T. J. and Martin, P. (2009) Wound repair at a glance. *Journal of Cell Science* 122 (18), 3209-3213.

Shi, H.-X., Lin, C., Lin, B.-B., Wang, Z.-G., Zhang, H.-Y., Wu, F.-Z., Cheng, Y., Xiang, L.-J., Guo, D.-J. and Luo, X. (2013) The anti-scar effects of basic fibroblast growth factor on the wound repair in vitro and in vivo. *PloS one* 8 (4), e59966.

Shin, J.-M., Yang, H.-W., Lee, S. A., Park, J.-H., Kim, H.-J. and Lee, H. M. Vitamin D inhibits TGF-β1-induced myofibroblast differentiation and extracellular matrix production via Smad2/3 signaling pathway in nasal polyp-derived fibroblasts. *Journal of Allergy and Clinical Immunology* 141 (2), AB271.

Singer, C. F., Kronsteiner, N., Marton, E., Kubista, M., Cullen, K. J., Hirtenlehner, K., Seifert, M. and Kubista, E. (2002) MMP-2 and MMP-9 expression in breast cancer-derived human fibroblasts is differentially regulated by stromal-epithelial interactions. *Breast cancer research and treatment* 72 (1), 69-77.

Sinno, H. and Prakash, S. (2013) Complements and the Wound Healing Cascade: An Updated Review. *Plastic Surgery International* 2013, 146764.

Sitrin, M. D., Bissonnette, M., Bolt, M. J., Wali, R., Khare, S., Scaglione-Sewell, B., Skarosi, S. and Brasitus, T. A. (1999) Rapid effects of 1,25(OH)2 vitamin D3 on signal transduction systems in colonic cells. *Steroids* 64 (1-2), 137-42.

Sitrin, M. D., Bissonnette, M., Bolt, M. J. G., Wali, R., Khare, S., Scaglione-Sewell, B., Skarosi, S. and Brasitus, T. A. (1999) Rapid effects of 1,25(OH)2 vitamin D3 on signal transduction systems in colonic cells. *Steroids* 64 (1), 137-142.

Slemp, A. E. and Kirschner, R. E. (2006) Keloids and scars: a review of keloids and scars, their pathogenesis, risk factors, and management. *Curr Opin Pediatr* 18 (4), 396-402.

Slominski, A., Kim, T.-K., Zmijewski, M. A., Janjetovic, Z., Li, W., Chen, J., Kusniatsova, E. I., Semak, I., Postlethwaite, A., Miller, D. D., Zjawiony, J. K. and Tuckey, R. C. (2013) Novel vitamin D photoproducts and their precursors in the skin. *Dermato-endocrinology* 5 (1), 7-19.

Slominski, A., Semak, I., Zjawiony, J., Wortsman, J., Li, W., Szczesniewski, A. and Tuckey, R. C. (2005) The cytochrome P450scc system opens an alternate pathway of vitamin D3 metabolism. *Febs j* 272 (16), 4080-90.

Slominski, A., Semak, I., Zjawiony, J., Wortsman, J., Li, W., Szczesniewski, A. and Tuckey, R. C. (2005) The cytochrome P450scc system opens an alternate pathway of vitamin D3 metabolism. *The FEBS journal* 272 (16), 4080-4090.

Slominski, A., Zjawiony, J., Wortsman, J., Semak, I., Stewart, J., Pisarchik, A., Sweatman, T., Marcos, J., Dunbar, C. and Tuckey, R. C. (2004) A novel pathway for sequential transformation of 7-dehydrocholesterol and expression of the P450scc system in mammalian skin. *European journal of biochemistry / FEBS* 271 (21), 4178-4188.

Slominski, A. T., Kim, T.-K., Hobrath, J. V., Janjetovic, Z., Oak, A. S., Postlethwaite, A., Lin, Z., Li, W., Takeda, Y. and Jetten, A. M. (2017) Characterization of a new pathway that activates lumisterol in vivo to biologically active hydroxylumisterols. *Scientific Reports* 7 (1), 11434.

Slominski, A. T., Kim, T.-K., Li, W. and Tuckey, R. C. (2016) Classical and non-classical metabolic transformation of vitamin D in dermal fibroblasts. *Experimental dermatology* 25 (3), 231-232.

Slominski, A. T., Kim, T.-K., Li, W., Yi, A.-K., Postlethwaite, A. and Tuckey, R. C. (2014) The role of CYP11A1 in the production of vitamin D metabolites and their role in the regulation of epidermal functions. *The Journal of steroid biochemistry and molecular biology* 144PA, 28-39.

Slominski, A. T., Kim, T. K., Li, W., Yi, A. K., Postlethwaite, A. and Tuckey, R. C. (2014) The role of CYP11A1 in the production of vitamin D metabolites and their role in the regulation of epidermal functions. *J Steroid Biochem Mol Biol* 144PA, 28-39.

Smith, R. S., Smith, T. J., Blieden, T. M. and Phipps, R. P. (1997) Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation. *Am J Pathol* 151 (2), 317-22.

Smithmyer, M. E., Sawicki, L. A. and Kloxin, A. M. (2014) Hydrogel scaffolds as in vitro models to study fibroblast activation in wound healing and disease. *Biomaterials Science* 2 (5), 634-650.

Smola, H., Thiekötter, G. and Fusenig, N. (1993) Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. *The Journal of Cell Biology* 122 (2), 417-429.

Snippert, H. J., Haegebarth, A., Kasper, M., Jaks, V., van Es, J. H., Barker, N., van de Wetering, M., van den Born, M., Begthel, H., Vries, R. G., Stange, D. E., Toftgård, R. and Clevers, H. (2010) Lgr6 Marks Stem

Cells in the Hair Follicle That Generate All Cell Lineages of the Skin. *Science* 327 (5971), 1385-1389.
Sollid, S. T., Hutchinson, M. Y. S., Berg, V., Fuskevag, O. M., Figenschau, Y., Thorsby, P. M. and Jorde, R. (2016) Effects of vitamin D binding protein phenotypes and vitamin D supplementation on serum total 25(OH)D and directly measured free 25(OH)D. *European journal of endocrinology / European Federation of Endocrine Societies* 174 (4), 445-52.
Sophors, P., Kim, Y. M., Seo, G. Y., Huh, J. S., Lim, Y., Koh, D. S. and Cho, M. (2016) A synthetic isoflavone, DCMF, promotes human keratinocyte migration by activating Src/FAK signaling pathway. *Biochem Biophys Res Commun* 472 (2), 332-8.
Sorrell, J. M. and Caplan, A. I. (2004) Fibroblast heterogeneity: more than skin deep. *Journal of cell science* 117 (5), 667-675.
Speeckaert, M., Huang, G., Delanghe, J. R. and Taes, Y. E. C. (2006) Biological and clinical aspects of the vitamin D binding protein (Gc-globulin) and its polymorphism. *Clinica Chimica Acta* 372 (1–2), 33-42.
St-Arnaud, R. (2011) CYP24A1: Structure, Function, and Physiological Role. In Feldman, D. (editor) *Vitamin D.* Vol. I.

edition. Elsevier.
Stadelmann, W. K., Digenis, A. G. and Tobin, G. R. (1998) Physiology and healing dynamics of chronic cutaneous wounds. *The American Journal of Surgery* 176 (2), 26S-38S.
Stadelmann, W. K., Digenis, A. G. and Tobin, G. R. (1998) Physiology and healing dynamics of chronic cutaneous wounds. *The American Journal of Surgery* 176 (2, Supplement 1), 26S-38S.
Stevenson, S., Nelson, L.D., Huq, S., Sharpe, D.T., and Thornton, M.J. (2005) Oestrogens and wound healing: migration, proliferation and secretion of paracrine factors by human dermal fibroblasts in vitro. *Endocrine Abstracts*, P80.
Stojadinovic, O. and Tomic-Canic, M. (2013) Human ex vivo wound healing model. *Methods Mol Biol* 1037, 255-64.
Su, W.-H., Cheng, M.-H., Lee, W.-L., Tsou, T.-S., Chang, W.-H., Chen, C.-S. and Wang, P.-H. (2010) Nonsteroidal anti-inflammatory drugs for wounds: pain relief or excessive scar formation? *Mediators of inflammation* 2010.
Sun, T. T., Eichner, R., Nelson, W. G., Tseng, S. C., Weiss, R. A., Jarvinen, M. and Woodcock-Mitchell, J. (1983) Keratin classes: molecular markers for different types of epithelial differentiation. *J Invest Dermatol* 81 (1 Suppl), 109s-15s.
Sundberg, J. P. and Nanney, L. (2012) *Skin and Adnexa*. *Comparative Anatomy and Histology.* Elsevier
Takahashi, K., Nakayama, Y., Horiuchi, H., Ohta, T., Komoriya, K., Ohmori, H. and Kamimura, T. (2002) Human neutrophils express messenger RNA of vitamin D receptor and respond to 1 α, 25-dihydroxyvitamin D3. *Immunopharmacology and immunotoxicology* 24 (3), 335-347.

Takeda, E., Kuroda, Y., Saijo, T., Naito, E., Kobashi, H., Yokota, I. and Miyao, M. (1987) 1α-Hydroxyvitamin D₃ Treatment of Three Patients With 1,25-Dihydroxyvitamin D-Receptor-Defect Rickets and Alopecia. *Pediatrics* 80 (1), 97-101.

Takeshita, A., Yen, P. M., Misiti, S., Cardona, G. R., Liu, Y. and Chin, W. W. (1996) Molecular cloning and properties of a full-length putative thyroid hormone receptor coactivator. *Endocrinology* 137 (8), 3594-3597.

Takeyama, K.-I., Masuhiro, Y., Fuse, H., Endoh, H., Murayama, A., Kitanaka, S., Suzawa, M., Yanagisawa, J. and Kato, S. (1999) Selective interaction of vitamin D receptor with transcriptional coactivators by a vitamin D analog. *Molecular and Cellular Biology* 19 (2), 1049-1055.

Tan, X., Li, Y. and Liu, Y. (2007) Therapeutic role and potential mechanisms of active vitamin D in renal interstitial fibrosis. *The Journal of steroid biochemistry and molecular biology* 103 (3-5), 491-496.

Tavera-Mendoza, L., Wang, T.-T., Lallemant, B., Zhang, R., Nagai, Y., Bourdeau, V., Ramirez-Calderon, M., Desbarats, J., Mader, S. and White, J. H. (2006) Convergence of vitamin D and retinoic acid signalling at a common hormone response element. *EMBO Reports* 7 (2), 180-185.

Teichert, A., Elalieh, H. and Bikle, D. (2010) Disruption of the Hedgehog Signaling Pathway Contributes to the Hair Follicle Cycling Deficiency in Vdr Knockout Mice. *Journal of cellular physiology* 225 (2), 482-489.

Thacher, T. D., Fischer, P. R., Singh, R. J., Roizen, J. and Levine, M. A. (2015) CYP2R1 Mutations Impair Generation of 25-hydroxyvitamin D and Cause an Atypical Form of Vitamin D Deficiency. *The Journal of Clinical Endocrinology and Metabolism* 100 (7), E1005-E1013.

Thangapazham, R. L., Darling, T. N. and Meyerle, J. (2014) Alteration of Skin Properties with Autologous Dermal Fibroblasts. *International Journal of Molecular Sciences* 15 (5), 8407-8427.

Thompson, P. D., Jurutka, P. W., Haussler, C. A., Whitfield, G. K. and Haussler, M. R. (1998) Heterodimeric DNA binding by the vitamin D receptor and retinoid X receptors is enhanced by 1,25-dihydroxyvitamin D3 and inhibited by 9-cis-retinoic acid. Evidence for allosteric receptor interactions. *J Biol Chem* 273 (14), 8483-91.

Thornton, D. M. J. (1990) *Androgen action in Cultured Dermal Papilla Cells Derived from Human Hair Follicles.* PhD. University of Bradford.

Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M. and Martin, G. R. (1979) Laminin--a glycoprotein from basement membranes. *J Biol Chem* 254 (19), 9933-7.

Tobin, D. J. (2006) Biochemistry of human skin-our brain on the outside. *Chemical Society Reviews* 35 (1), 52-67.

Tokar, E. J. and Webber, M. M. (2005) Cholecalciferol (vitamin D 3) inhibits growth and invasion by up-regulating nuclear receptors and 25-hydroxylase (CYP27A1) in human prostate cancer cells. *Clinical & experimental metastasis* 22 (3), 275-284.

Torma, H., Rollman, O., Binderup, L. and Michaelsson, G. (1996) Vitamin D analogs affect the uptake and metabolism of retinol by human epidermal keratinocytes in culture. *J Investig Dermatol Symp Proc* 1 (1), 49-53.

Trang, H. M., Cole, D. E., Rubin, L. A., Pierratos, A., Siu, S. and Vieth, R. (1998) Evidence that vitamin D3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D2. *Am J Clin Nutr* 68 (4), 854-8.

Trémezaygues, L. and Reichrath, J. (2011) Vitamin D analogs in the treatment of psoriasis: Where are we standing and where will we be going? *Dermato-endocrinology* 3 (3), 180-186.

Tripkovic, L., Wilson, L. R., Hart, K., Elliott, R., Smith, C. P., Bucca, G., Penson, S., Chope, G., Hypponen, E., Berry, J. and Lanham-New, S. (2014) DAILY SUPPLEMENTATION WITH VITAMIN D3 IS COMPREHENSIVELY MORE EFFECTIVE THAN VITAMIN D2 IN RAISING 25OHD STATUS AND CONCOMITANTLY REDUCING PARATHYROID HORMONE LEVELS: IMPLICATIONS FOR BONE HEALTH. *Osteoporosis International* 25, S665-S665.

Trochoutsou, A. I., Kloukina, V., Samitas, K. and Xanthou, G. (2015) *Vitamin-D in the Immune System: Genomic and Non-Genomic Actions.* Vol. 15.

Trost, J. G., Applebaum, D. S. and Orengo, I. (2016) Common Adult Skin and Soft Tissue Lesions. *Seminars in Plastic Surgery* 30 (3), 98-107.

Trounson, A. (2006) The Production and Directed Differentiation of Human Embryonic Stem Cells. *Endocrine Reviews* 27 (2), 208-219.

Tuckey, R. C., Li, W., Shehabi, H. Z., Janjetovic, Z., Nguyen, M. N., Kim, T. K., Chen, J., Howell, D. E., Benson, H. A., Sweatman, T., Baldisseri, D. M. and Slominski, A. (2011) Production of 22-hydroxy metabolites of vitamin d3 by cytochrome p450scc (CYP11A1) and analysis of their biological activities on skin cells. *Drug Metab Dispos* 39 (9), 1577-88.

Tuckey, R. C., Li, W., Zjawiony, J. K., Zmijewski, M. A., Nguyen, M. N., Sweatman, T., Miller, D. and Slominski, A. (2008) Pathways and products for the metabolism of vitamin D3 by cytochrome P450scc. *Febs j* 275 (10), 2585-96.

Tuohimaa, P. (2009) Vitamin D and aging. *The Journal of steroid biochemistry and molecular biology* 114 (1-2), 78-84.

Vaananen, A., Tjaderhane, L., Eklund, L., Heljasvaara, R., Pihlajaniemi, T., Herva, R., Ding, Y., Bartlett, J. D. and Salo, T. (2004) Expression of collagen XVIII and MMP-20 in developing teeth and odontogenic tumors. *Matrix Biol* 23 (3), 153-61.

Vagnozzi, A. N., Reiter, J. F. and Wong, S. Y. (2015) Hair follicle and interfollicular epidermal stem cells make varying contributions to wound regeneration. *Cell Cycle* 14 (21), 3408-17.

Vaid, M., Prasad, R., Sun, Q. and Katiyar, S. K. (2011) Silymarin Targets β-Catenin Signaling in Blocking Migration/Invasion of Human Melanoma Cells. *PLoS ONE* 6 (7), e23000.

Väisänen, S., Dunlop, T. W., Sinkkonen, L., Frank, C. and Carlberg, C. (2005) Spatio-temporal activation of chromatin on the human CYP24 gene promoter in the presence of 1α, 25-dihydroxyvitamin D3. *Journal of molecular biology* 350 (1), 65-77.

van Etten, E., Stoffels, K., Gysemans, C., Mathieu, C. and Overbergh, L. (2008) Regulation of vitamin D homeostasis: implications for the immune system. *Nutr Rev* 66 (10 Suppl 2), S125-34.

Van Etten, E., Stoffels, K., Gysemans, C., Mathieu, C. and Overbergh, L. (2008) Regulation of vitamin D homeostasis: implications for the immune system. *Nutrition reviews* 66 (suppl 2), S125-S134.

Van Linthout, S., Miteva, K. and Tschöpe, C. (2014) Crosstalk between fibroblasts and inflammatory cells. *Cardiovascular Research* 102 (2), 258-269.

Vanhaesebroeck, B., Jones, G. E., Allen, W. E., Zicha, D., Hooshmand-Rad, R., Sawyer, C., Wells, C., Waterfield, M. D. and Ridley, A. J. (1999) Distinct PI(3)Ks mediate mitogenic signalling and cell migration in macrophages. *Nat Cell Biol* 1 (1), 69-71.

Vantieghem, K. (2006) *Photoproduction of Vitamin D_3 and Activation into 1a, 25-dihydroxyvitamin D3 in Human Epidermal Keratinocytes, Dermal Fibroblasts and Other Cells.* Leuven University Press.

Vantieghem, K., De Haes, P., Bouillon, R. and Segaert, S. (2006) Dermal fibroblasts pretreated with a sterol Delta7-reductase inhibitor produce 25-hydroxyvitamin D3 upon UVB irradiation. *J Photochem Photobiol B* 85 (1), 72-8.

Varani, J., Kang, S., Stoll, S. and Elder, J. T. (1998) Human psoriatic skin in organ culture: comparison with normal skin exposed to exogenous growth factors and effects of an antibody to the EGF receptor. *Pathobiology* 66 (6), 253-9.

Volk, S. W., Wang, Y., Mauldin, E. A., Liechty, K. W. and Adams, S. L. (2011) Diminished type III collagen promotes myofibroblast differentiation and increases scar deposition in cutaneous wound healing. *Cells Tissues Organs* 194 (1), 25-37.

Volk, S. W., Wang, Y., Mauldin, E. A., Liechty, K. W. and Adams, S. L. (2011) Diminished Type III Collagen Promotes Myofibroblast Differentiation and Increases Scar Deposition in Cutaneous Wound Healing. *Cells, Tissues, Organs* 194 (1), 25-37.

Vuolo, L., Di Somma, C., Faggiano, A. and Colao, A. (2012) Vitamin D and cancer.

Waelti, E. R., Inaebnit, S. P., Rast, H. P., Hunziker, T., Limat, A., Braathen, L. R. and Wiesmann, U. (1992) Co-culture of human keratinocytes on post-mitotic human dermal fibroblast feeder cells: production of large amounts of interleukin 6. *J Invest Dermatol* 98 (5), 805-8.

Wang, L.-F., Tai, C.-F., Chien, C.-Y., Chiang, F.-Y. and Chen, J. Y.-F. (2015) Vitamin D decreases the secretion of matrix metalloproteinase-2 and matrix metalloproteinase-9 in fibroblasts derived from Taiwanese patients with chronic rhinosinusitis with nasal polyposis. *The Kaohsiung Journal of Medical Sciences* 31 (5), 235-240.

Wang, L. F., Tai, C. F., Chien, C. Y., Chiang, F. Y. and Chen, J. Y. (2015) Vitamin D decreases the secretion of matrix metalloproteinase-2 and matrix metalloproteinase-9 in fibroblasts derived from Taiwanese patients with chronic rhinosinusitis with nasal polyposis. *Kaohsiung J Med Sci* 31 (5), 235-40.

Wang, T.-T., Nestel, F. P., Bourdeau, V., Nagai, Y., Wang, Q., Liao, J., Tavera-Mendoza, L., Lin, R., Hanrahan, J. W., Mader, S. and White, J. H. (2004)

Cutting Edge: 1,25-Dihydroxyvitamin D₃ Is a Direct Inducer of Antimicrobial Peptide Gene Expression. *The Journal of Immunology* 173 (5), 2909-2912.

Wang, Z., Lin, Y. S., Dickmann, L. J., Poulton, E. J., Eaton, D. L., Lampe, J. W., Shen, D. D., Davis, C. L., Shuhart, M. C. and Thummel, K. E. (2013) Enhancement of hepatic 4-hydroxylation of 25-hydroxyvitamin D3 through CYP3A4 induction in vitro and in vivo: implications for drug-induced osteomalacia. *J Bone Miner Res* 28 (5), 1101-16.

Wang, Z., Lin, Y. S., Zheng, X. E., Senn, T., Hashizume, T., Scian, M., Dickmann, L. J., Nelson, S. D., Baillie, T. A. and Hebert, M. F. (2012) An inducible cytochrome P450 3A4-dependent vitamin D catabolic pathway. *Molecular pharmacology* 81 (4), 498-509.

Wang, Z., Schuetz, E. G., Xu, Y. and Thummel, K. E. (2013) Interplay between Vitamin D and the Drug Metabolizing Enzyme CYP3A4. *The Journal of steroid biochemistry and molecular biology* 136, 54-58.

Wang, Z., Wang, Y., Farhangfar, F., Zimmer, M. and Zhang, Y. (2012) Enhanced Keratinocyte Proliferation and Migration in Co-culture with Fibroblasts. *PLOS ONE* 7 (7), e40951.

Watabe, H., Soma, Y., Kawa, Y., Ito, M., Ooka, S., Ohsumi, K., Baba, T., Kawakami, T., Hosaka, E. and Kimura, S. (2002) Differentiation of murine melanocyte precursors induced by 1, 25-dihydroxyvitamin D3 is associated with the stimulation of endothelin B receptor expression. *Journal of investigative dermatology* 119 (3), 583-589.

Watt, F. M. and Jensen, K. B. (2009) Epidermal stem cell diversity and quiescence. *EMBO Molecular Medicine* 1 (5), 260-267.

Webb, A. H., Gao, B. T., Goldsmith, Z. K., Irvine, A. S., Saleh, N., Lee, R. P., Lendermon, J. B., Bheemreddy, R., Zhang, Q., Brennan, R. C., Johnson, D., Steinle, J. J., Wilson, M. W. and Morales-Tirado, V. M. (2017) Inhibition of MMP-2 and MMP-9 decreases cellular migration, and angiogenesis in in vitro models of retinoblastoma. *BMC Cancer* 17 (1), 434.

Wei, C., Wang, X., Chen, M., Ouyang, K., Song, L.-S. and Cheng, H. (2008) Calcium flickers steer cell migration. *Nature* 457, 901.

Wei, C., Wang, X., Chen, M., Ouyang, K., Song, L. S. and Cheng, H. (2009) Calcium flickers steer cell migration. *Nature* 457 (7231), 901-5.

Wei Zheng, D. T. (2013) Vitamin D. In Janos Zempleni, J. W. S. (editor) *Handbook of Vitamins.* 5th Edition edition. CRC Press.

Werb, Z. and Aggeler, J. (1978) Proteases induce secretion of collagenase and plasminogen activator by fibroblasts. *Proc Natl Acad Sci U S A* 75 (4), 1839-43.

Werner, S. (1998) Keratinocyte growth factor: a unique player in epithelial repair processes. *Cytokine Growth Factor Rev* 9 (2), 153-65.

Werner, S. and Grose, R. (2003) Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 83 (3), 835-70.

Werner, S. and Smola, H. (2001) Paracrine regulation of keratinocyte proliferation and differentiation. *Trends Cell Biol* 11 (4), 143-6.

Whiting, S. J. and Calvo, M. S. (2013) Chapter 43 - Current Understanding of Vitamin D Metabolism, Nutritional Status, and Role in Disease Prevention1 A2 - Coulston, Ann M. In Boushey, C. J. and Ferruzzi, M. G. (editors) *Nutrition in the Prevention and Treatment of Disease (Third Edition).* Academic Press. 811-837.

Wilson, L. R., Tripkovic, L., Hart, K., Elliott, R., Smith, C. P., Bucca, G., Penson, S., Chope, G., Hypponen, E., Berry, J. and Lanham-New, S. (2014) IS VITAMIN D3 MORE EFFECTIVE THAN VITAMIN D2 IN RAISING 25OHD STATUS IN WOMEN WITH OSTEOPOROSIS AND OSTEOPENIA? *Osteoporosis International* 25, S687-S687.

Witte, R. P. and Kao, W. J. (2005) Keratinocyte-fibroblast paracrine interaction: the effects of substrate and culture condition. *Biomaterials* 26 (17), 3673-82.

Wong, P. and Coulombe, P. A. (2003) Loss of keratin 6 (K6) proteins reveals a function for intermediate filaments during wound repair. *The Journal of Cell Biology* 163 (2), 327-337.

Wu, X. U. E., Yang, L., Zheng, Z., Li, Z., Shi, J., Li, Y. A. N., Han, S., Gao, J., Tang, C., Su, L. and Hu, D. (2016) Src promotes cutaneous wound healing by regulating MMP-2 through the ERK pathway. *International Journal of Molecular Medicine* 37 (3), 639-648.

Wysocki, A. B., Staiano-Coico, L. and Grinnell, F. (1993) Wound Fluid from Chronic Leg Ulcers Contains Elevated Levels of Metalloproteinases MMP-2 and MMP-9. *Journal of Investigative Dermatology* 101 (1), 64-68.

Xu, F., Zhang, C. and Graves, D. T. (2013) Abnormal cell responses and role of TNF-in impaired diabetic wound healing. *BioMed research international* 2013.

Xue, M. and Jackson, C. J. (2015) Extracellular Matrix Reorganization During Wound Healing and Its Impact on Abnormal Scarring. *Advances in Wound Care* 4 (3), 119-136.

Xue, M., Le, N. T. and Jackson, C. J. (2006) Targeting matrix metalloproteases to improve cutaneous wound healing. *Expert Opin Ther Targets* 10 (1), 143-55.

Yamamoto, N. and Naraparaju, V. R. (1996) Role of vitamin D3-binding protein in activation of mouse macrophages. *J Immunol* 157 (4), 1744-9.

Yamauchi, J., Sekiguchi, M., Shirai, T. and Ishimi, Y. (2012) Vitamin D Receptor Is Not Essential for Extracellular Signal-Related Kinase Phosphorylation by Vitamin D3 in Human Caco-2/TC7 Cells. *Bioscience, Biotechnology, and Biochemistry* 76 (8), 1588-1590.

Yan, C., Grimm, W. A., Garner, W. L., Qin, L., Travis, T., Tan, N. and Han, Y.-P. (2010) Epithelial to Mesenchymal Transition in Human Skin Wound Healing Is Induced by Tumor Necrosis Factor-α through Bone Morphogenic Protein-2. *The American Journal of Pathology* 176 (5), 2247-2258.

Yang, C.-C. and Cotsarelis, G. (2010) Review of hair follicle dermal cells. *Journal of dermatological science* 57 (1), 2.

Yang, S. and Huang, X.-Y. (2005) Ca2+ influx through L-type Ca2+ channels controls the trailing tail contraction in growth factor-induced fibroblast cell migration. *Journal of Biological Chemistry* 280 (29), 27130-27137.

Yang, S. and Huang, X. Y. (2005) Ca2+ influx through L-type Ca2+ channels controls the trailing tail contraction in growth factor-induced fibroblast cell migration. *J Biol Chem* 280 (29), 27130-7.

Yannas, I. V. (2000) Synthesis of organs: In vitro or in vivo? *Proceedings of the National Academy of Sciences* 97 (17), 9354-9356.

Yao, T. P., Ku, G., Zhou, N., Scully, R. and Livingston, D. M. (1996) The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. *Proceedings of the National Academy of Sciences* 93 (20), 10626-10631.

Yasmin, R., Williams, R. M., Xu, M. and Noy, N. (2005) Nuclear import of the retinoid X receptor, the vitamin D receptor, and their mutual heterodimer. *Journal of Biological Chemistry* 280 (48), 40152-40160.

Yasmin, R., Williams, R. M., Xu, M. and Noy, N. (2005) Nuclear import of the retinoid X receptor, the vitamin D receptor, and their mutual heterodimer. *J Biol Chem* 280 (48), 40152-60.

Yates, S. and Rayner, T. E. (2002) Transcription factor activation in response to cutaneous injury: role of AP-1 in reepithelialization. *Wound Repair Regen* 10 (1), 5-15.

Yin, K. and Agrawal, D. K. (2014) Vitamin D and inflammatory diseases. *Journal of Inflammation Research* 7, 69-87.

Ying, H.-Q., Sun, H.-L., He, B.-S., Pan, Y.-Q., Wang, F., Deng, Q.-W., Chen, J., Liu, X. and Wang, S.-K. (2015) Circulating vitamin D binding protein, total, free and bioavailable 25-hydroxyvitamin D and risk of colorectal cancer. *Scientific reports* 5, 7956.

Yoshinaga, Y., Higaki, M., Terajima, S., Ohkubo, E., Nogita, T., Miyasaka, N. and Kawashima, M. (1995) Detection of inflammatory cytokines in psoriatic skin. *Arch Dermatol Res* 287 (2), 158-64.

Yousefzadeh, P., Shapses, S. A. and Wang, X. (2014) Vitamin D Binding Protein Impact on 25-Hydroxyvitamin D Levels under Different Physiologic and Pathologic Conditions. *International Journal of Endocrinology* 2014, 6.

Zehnder, D., Bland, R., Williams, M. C., McNinch, R. W., Howie, A. J., Stewart, P. M. and Hewison, M. (2001) Extrarenal Expression of 25-Hydroxyvitamin D3-1α-Hydroxylase. *The Journal of Clinical Endocrinology & Metabolism* 86 (2), 888-894.

Zella, L. A., Shevde, N. K., Hollis, B. W., Cooke, N. E. and Pike, J. W. (2008) Vitamin D-binding protein influences total circulating levels of 1,25-dihydroxyvitamin D3 but does not directly modulate the bioactive levels of the hormone in vivo. *Endocrinology* 149 (7), 3656-67.

Zenz, R., Eferl, R., Scheinecker, C., Redlich, K., Smolen, J., Schonthaler, H. B., Kenner, L., Tschachler, E. and Wagner, E. F. (2008) Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. *Arthritis Research & Therapy* 10 (1), 201-201.

Zerr, P., Vollath, S., Palumbo-Zerr, K., Tomcik, M., Huang, J., Distler, A., Beyer, C., Dees, C., Gela, K., Distler, O., Schett, G. and Distler, J. H. W. (2015)

Vitamin D receptor regulates TGF-β signalling in systemic sclerosis. *Annals of the Rheumatic Diseases* 74 (3), e20.

Zhang, C., Baudino, T. A., Dowd, D. R., Tokumaru, H., Wang, W. and MacDonald, P. N. (2001) Ternary complexes and cooperative interplay between NCoA-62/Ski-interacting protein and steroid receptor coactivators in vitamin D receptor-mediated transcription. *Journal of Biological Chemistry* 276 (44), 40614-40620.

Zhang, C., Baudino, T. A., Dowd, D. R., Tokumaru, H., Wang, W. and MacDonald, P. N. (2001) Ternary complexes and cooperative interplay between NCoA-62/Ski-interacting protein and steroid receptor coactivators in vitamin D receptor-mediated transcription. *J Biol Chem* 276 (44), 40614-20.

Zhang, J., Chalmers, M. J., Stayrook, K. R., Burris, L. L., Wang, Y., Busby, S. A., Pascal, B. D., Garcia-Ordonez, R. D., Bruning, J. B., Istrate, M. A., Kojetin, D. J., Dodge, J. A., Burris, T. P. and Griffin, P. R. (2011) DNA binding alters coactivator interaction surfaces of the intact VDR–RXR complex. *Nature structural & molecular biology* 18 (5), 556-563.

Zhang, Z., Yu, X., Fang, X., Liang, A., Yu, Z., Gu, P., Zeng, Y., He, J., Zhu, H. and Li, S. (2015) Preventive effects of vitamin D treatment on bleomycin-induced pulmonary fibrosis. *Scientific reports* 5.

Zhao, W., Wang, X., Sun, K.-H. and Zhou, L. (2018) α-smooth muscle actin is not a marker of fibrogenic cell activity in skeletal muscle fibrosis. *PLoS ONE* 13 (1), e0191031.

Zhao, X.-K., Cheng, Y., Liang Cheng, M., Yu, L., Mu, M., Li, H., Liu, Y., Zhang, B., Yao, Y., Guo, H., Wang, R. and Zhang, Q. (2016) Focal Adhesion Kinase Regulates Fibroblast Migration via Integrin beta-1 and Plays a Central Role in Fibrosis. *Scientific Reports* 6, 19276.

Zhu, J. G., Ochalek, J. T., Kaufmann, M., Jones, G. and DeLuca, H. F. (2013) CYP2R1 is a major, but not exclusive, contributor to 25-hydroxyvitamin D production in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 110 (39), 15650-15655.

Zierold, C., Darwish, H. M. and DeLuca, H. F. (1995) Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter. *J Biol Chem* 270 (4), 1675-8.

9 APPENDIX

9.1 Gelatine zymography solutions preparation

9.1.1 Gelatine zymography reagents and solutions

Separating Gel Buffer (1.5M Tris Base)

- 90.86g Tris-base, final concentration 1.5M
- 275 ml distilled water
- The Tris base was dissolved in 275 ml distilled water and made up to 500 ml solution, the pH was adjusted to 8.8, and stored at 4ºC.

Stacking Gel Buffer (0.5M Tris base)

- 6g Tris base, final concentration 0.5M
- 60 ml distilled water
- The Tris base was dissolved in 60 ml distilled water and made up to 100ml solution, the pH was adjusted to 6.8 and stored at 4ºC.

Running Buffer

- 15g Tris base, final concentration 25 mM
- 72g Glycine, final concentration 200 mM
- 5g SDS, final concentration 0.5% w/v
- The Tris base, glycine and SDS were dissolved in 1L distilled water and was stored at 4ºC. The prepared solution was diluted using the ratio 280 ml distilled water plus 70 ml running buffer when used.

2x Non-reducing Sample Buffer

- 3.125 ml (0.5 M Tris base, pH 6.8) Stacking gel buffer, final concentration 62.5 mM
- 10 ml 10% SDS, final concentration 40% v/v
- 10 ml glycerol, final concentration 50% v/v
- 1.87 ml distilled water

- 125 µl Bromophenol Blue
- All components were mixed gently and the final solution was stored at room temperature.

12.5x Washing Buffer

- 125 ml Triton X-100
- 875 ml distilled water
- The 125 ml Triton X-100 was added as 5 parts until full dissolved and stored at room temperature. The solution was diluted as 200 ml distilled water plus 50 ml Triton X-100 when used.

10x Incubation Buffer

- 78.8g Tris base, final concentration 50 mM
- 14.7g $CaCl_2 \bullet 2H_2O$, final concentration 100 mM
- 29.2g NaCl, final concentration 50 mM
- 5 ml Brij-35, final concentration 0.5% v/v
- All components were dissolved in 1L distilled water, the pH was adjusted at 7.6, and stored at 4ºC. The solution was diluted as 180 ml distilled water plus 20 ml incubation buffer when used.

Coomassie Blue Staining Solution

- 0.2g Coomassie brilliant blue R-250, final concentration 0.02% v/v
- 100 ml glacial acetic acid, final concentration 10% v/v
- 250 ml methanol, final concentration 25% v/v
- All components were dissolved in 1L distilled water with magnetic stirring overnight, and the solution was stored at room temperature.

Destain Solution

- 50 ml Methanol, final concentration 5% v/v
- 50 ml Glacial acetic acid, final concentration 5% v/v

- The Methanol and Glacial acetic acid were dissolved in 1L distilled water, and was stored at room temperature.

10% SDS Solution

- 10g SDS
- 80 ml distilled water
- The SDS was dissolved in 80 ml distilled water and made up to 100 ml distilled water, and when fully dissolved, was stored at room temperature.

10% APS Solution

- 0.12g ammonium persulfate (APS)
- 1.2 ml distilled water
- The APS dissolved in 1.2 ml distilled water, and was stored at -20ºC, until used.

9.2 $1,25(OH)_2D_3$ down-regulates α-SMA protein expression in scratched human dermal fibroblasts and the effect was dose-dependent

Western blotting confirmed that 10nM and 100nM $1,25(OH)_2D_3$ down-regulated protein expression of α-SMA in scratched human dermal fibroblasts after a 24 hour incubation.

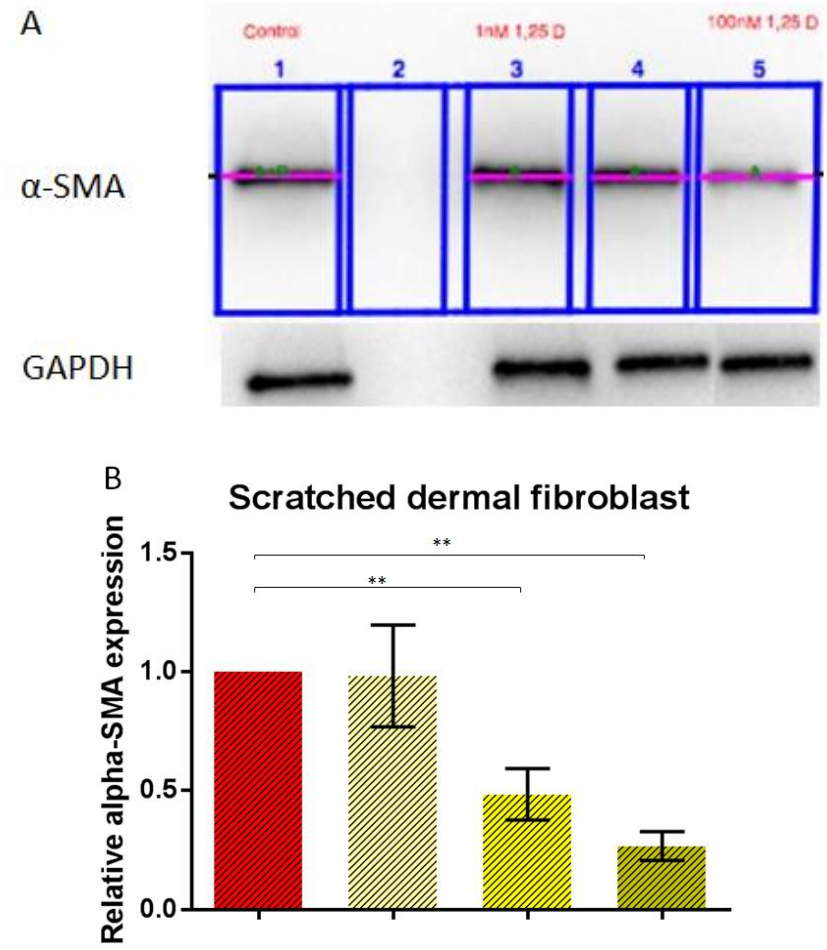


Figure 9-1: α-SMA expression by scratched dermal fibroblast after 24 hours incubation with $1,25(OH)_2D_3$. (A) Western Blot image of α-SMA expression by fibroblasts after incubation with vehicle control (0.01% ethanol), 1nM $1,25(OH)_2D_3$, 10nM $1,25(OH)_2D_3$ and 100nM $1,25(OH)_2D_3$; (B) Relative alpha-SMA protein expression in scratched dermal fibroblasts after 24 hours, quantitated by imageJ. Relative protein expression was normalised against vehicle control. Data presented as mean of three readings ± SEM. ** Denotes $p<0.01$ using one-way ANOVA.